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Brief Article

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Plant Growth Regulator Daminozide Is a Selective Inhibitor of Human KDM2/7 Histone Demethylases

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Supporting Information

ABSTRACT: The JmjC oxygenases catalyze the *N*-demethylation of N^{ϵ}-methyl lysine residues in histones and are current therapeutic targets. A set of human 2-oxoglutarate analogues were screened using a unified assay platform for JmjC demethylases and related oxygenases. Results led to the finding that daminozide (*N*-(dimethylamino)succinamic acid, 160 Da), a plant growth regulator, selectively inhibits the KDM2/7 JmjC subfamily. Kinetic and crystallographic studies reveal that daminozide chelates the active site metal via its hydrazide carbonyl and dimethylamino groups.

INTRODUCTION

Histone modifications are central to the regulation of eukaryotic gene expression. The dynamic methylation of lysine and arginine residues in histones has diverse transcriptional outcomes, with different methylation sites and states being associated with promotion or repression of transcription. There are two classes of histone lysine demethylases (KDMs), the largest of which uses 2-oxoglutarate (2OG) as a cosubstrate (the JmjC enzymes) and comprises ∼18 human enzymes grouped into five subfamilies (Figure 1). Several JmjC demethylases are targets for the treatment of diseases including leukemia, breast, and prostate cancers[1,2] and inflammation.[3] However, only limited inhibition data have been reported for the JmjC enzymes with few reports of selective inhibitors.[4−8]

2OG oxygenases have been targeted for therapeutic and agricultural applications:[9] an inhibitor of γ-butyrobetaine hydroxylase, BBOX1, is used clinically as a "cardioprotectant", inhibition of the collagen prolyl hydroxylases has been investigated for treating fibrotic disease, and inhibitors of the hypoxia inducible factor hydroxylases are in clinical trials for the treatment of anemia. These examples provide evidence that 2OG oxygenases are viable targets for therapeutic inhibition by small molecules in vivo. 2OG oxygenases involved in the biosynthesis of gibberellins, plant hormones involved in growth regulation, have also been targeted using small molecule inhibitors for agricultural and horticultural applications. Here we report that daminozide, *N*-(dimethylamino)succinamic acid, which was once widely used as a plant growth retardant but later withdrawn because of toxicity concerns, is a highly selective inhibitor of the KDM2/7 family of human JmjC histone demethylases.

RESULTS

To enable the identification of selective JmjC demethylase subfamily inhibitors, we developed a unified screening platform containing representatives of each of the five human demethylase subfamilies (KDM2A/FBXL11, KDM3A/JMJD1A, KDM4E/JMJD2E, KDM5C/JARID1C, KDM6B/JMJD3) (Supporting Information). Active enzymes were expressed using bacterial or eukaryotic expression systems and purified to near homogeneity (Supporting Information). Kinetic parameters for 2OG and histone fragment substrates were determined (Table 1), and activity AlphaScreens (amplified luminescence proximity homogeneous assays) were developed (Figure S1).[10] This assay is based on immunodetection of the lysine methylation state of biotin-conjugated histone peptide product, using streptavidin- and protein A-

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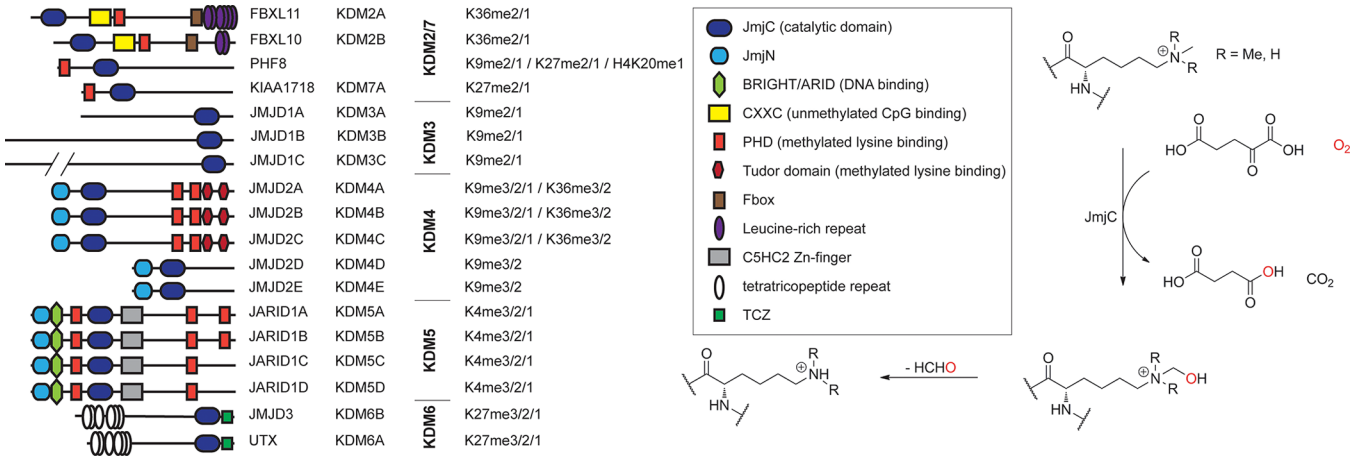

Figure 1. Subfamilies of the human 2-oxoglutarate (JmjC) histone demethylases, showing their domain architecture. Not all proposed family members are shown. Each demethylation reaction is coupled to 2OG decarboxylation and formaldehyde production. KIAA1718 has recently been assigned as KDM7A;[11] however, its JmjC domain shows high sequence and structural similarity with the KDM2 subfamily members.

Table 1. Inhibition Data (IC_{50}) for Daminozide and Its Analogues across KDMs and Other 2OG Oxygenases[a]

	Daminozide	22	23	24	25	26	27	28	29	30
KDM2A	1.5	63	0.37	0.25	0.43	3.7	>100	0.57	9.1	>100
PHF8	0.55	35	3.4	0.48	1.7	11.5	>100	2.3	6.9	117
KDM7A	(2.1)	-	-	-	-	-	-	-	-	-
KDM3A	>100	>100	>100	>100	3.0	>100	>100	12.7	>100	>100
KDM4E	>100	>100	4.3	0.2	0.4	>100	>100	23.9	>100	>100
KDM5C	>100	>100	1.1	0.48	1.9	>100	>100	4.5	42	>100
KDM6B	>100	>100	>100	>100	1.1	>100	>100	16.5	41.7	>100
FIH	(>100)	(>100)	(>100)	(>100)	(>100)	(>100)	(>100)	(>100)	(>100)	(>100)
PHD2	>100	>100	14.2	>100	19.6	>100	>100	>100	6.3	>100
BBOX1*	>100	>100	>100	>100	>100	>100	>100	>100	>100	>100

[a]Data are reported as IC_{50} in μM. AlphaScreen was used for all IC_{50} determinations except where values are given in parenthesis where a MALDI assay was used. All BBOX assays were run using a fluorescence based assay.[12] AlphaScreen assays were optimized to run at the linear range of the reaction. Assays were performed at concentrations of 2OG near experimentally determined 2OG K_m^{app} values. Where K_m^{app} values for 2OG for enzymes were unknown, the kinetic parameter was determined by using the FDH assay: KDM2A (12.5 ± 1.4 μM), PHF8 (16.6 ± 1.8 μM), KDM3A (5.3 ± 2.2 μM), KDM5C (41.7 ± 6.3 μM), and KDM6B (5.4 ± 0.4 μM).

conjugated beads for quantification. For counterscreening, we selected three clinically relevant oxygenases, the hypoxic response oxygenases PHD2 and FIH (prolyl and asparaginyl hydroxylases acting on hypoxia inducible factor, respectively), and BBOX1 (γ-butyrobetaine hydroxylase) for which AlphaScreen or fluorescence assays[12] were also developed. This screening platform is a significant improvement on previous methods for human 2OG oxygenases;[9] it enables the oxygenases to be used at low nanomolar concentrations. Standardization of the analytical methods for enzymes employing different types of substrates enabled quantitative comparisons of inhibitor potencies to be made.

In work aimed at identifying inhibitor scaffolds for the JmjC histone demethylases, we assembled a set of 2OG analogues known to inhibit 2OG oxygenases (Figure 2) and screened these against the 2OG oxygenase panel using the assay platform. As expected, known "generic" 2OG oxygenase inhibitors[9] including pyridine 2,4-dicarboxylic acid (**11**) and *N*-oxalylglycine (**12**) showed inhibition across the 2OG oxygenases tested, validating this assay platform. Interestingly, the clinically used histone deacetylase (HDAC) inhibitor Vorinostat/SAHA (suberoylanilide hydroxamic acid, **15**) inhibited several histone demethylases (Figure 2).

Unexpectedly, the plant growth regulator daminozide (**19**) stood out as a selective inhibitor of KDM2A (IC_{50} = 1.5 μM), which is selective for dimethyl lysine residues. Daminozide was identified as a plant growth retardant in the 1960s[14] and used to control stem growth, plant size, and fruit ripening for over 20 years.[15] In vivo, daminozide is proposed to inhibit 2OG oxygenases involved in gibberellin, and possibly ethylene, biosynthesis in plants.[16,17] However, daminozide usage in food crops was curtailed because of the potential carcinogenicity of its metabolite, 1,1-dimethylhydrazine (although is still used for ornamental plants).[18−20] A formaldehyde dehydrogenase (FDH) coupled assay monitoring formaldehyde production confirmed the potency of daminozide against KDM2A (IC_{50} = 1.5 μM). To test further for subfamily selectivity, daminozide was screened against two other members of the KDM2/7 subfamily (PHF8 and KDM7A, Table 1) which are structurally highly related to KDM2A and KDM2B and are also selective

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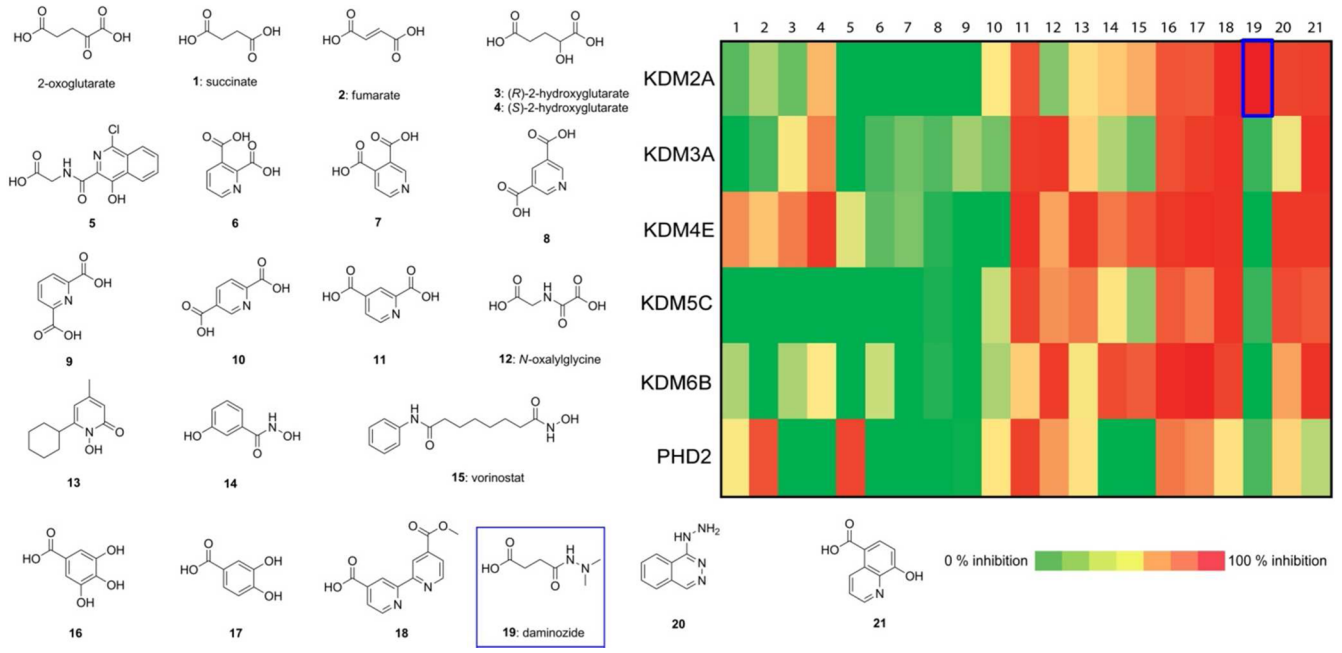

Figure 2. Heat map of JmjC demethylase inhibition by a set of 2OG analogues. Daminozide **19** is selective for KDM2A. Tricarboxylic acid cycle intermediates succinate **1** and fumarate **2** were generally poor demethylase inhibitors, though KDM4E[7] was an exception to this trend. The (*R*)- and (*S*)-2-hydroxyglutarate enantiomers, produced by gain-of-function mutations to isocitrate dehydrogenase, were inhibitors of the KDM2, KDM3, and KDM4 histone demethylases.[13] Catechols **16** and **17**, bipyridyl **18**, and 8-hydroxyquinoline **21** inhibited all demethylases screened. By contrast, **5** most potently inhibited PHD2 (prolyl hydroxylase domain enzyme isoform 2). Each compound was screened in an AlphaScreen assay and is represented as % inhibition of the enzyme at 20 μM compound. Details of the assays are in the Supporting Information.

for demethylation of dimethyl lysine residues but have different sequence specificities.[21−23] The results indicate that daminozide is at least 60-fold selective as an inhibitor of the KDM2/7 subfamily over the other demethylase subfamily members tested, with IC_{50} of 2 μM or less against KDM2A, PHF8, and KIAA1718 and IC_{50} of 127 μM for KDM3A (a different subfamily of dimethyl lysine demethylase) or greater (millimolar range) against other demethylases. No inhibition was observed (at 1 mM) against other biologically important 2OG oxygenases that catalyze hydroxylation, i.e., PHD2, FIH, and BBOX1. Given its simple achiral structure and low molecular weight (160 Da), the degree of selectivity exhibited by daminozide is remarkable.

Kinetic analyses revealed that daminozide is predominantly a competitive inhibitor with respect to 2OG (K_i = 1.97 μM) for KDM2A but shows mixed inhibition with respect to peptide substrate, binding predominantly to the enzyme-peptide complex (K_i = 85 μM, α = 0.13) (Figure 3). The latter observation is notable given that daminozide contains a dimethylamino group as do the KDM2/7 subfamily dimethyl

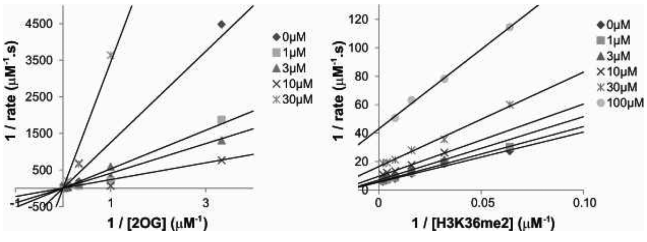

Figure 3. Mode of inhibition of the KDM2/7 subfamily by daminozide. Inhibition of KDM2A by daminozide is competitive with 2OG but not peptide substrate.

lysine substrates and might thus be expected to compete with the dimethylated lysine substrate. The pK_a of the daminozide hydrazide amine is 2.8 while that of its carboxylate is 4.6,[24] suggesting that it may bind the active site iron; NMR analyses show daminozide complexes to Fe(II) in solution (Figure S2).

We then investigated the structural basis of the selective inhibition by daminozide on the KDM2/7 subfamily. We obtained structures of daminozide complexed with two demethylases, PHF8 and KDM4A, and a hydroxylase, FIH (Figure 4). The structures support the proposed general mode of inhibition by daminozide, i.e., by binding in the 2OG binding pocket and chelating to the active site metal via its acylhydrazide carbonyl and dimethylamino groups (Figure 4). While the coordinate position of the daminozide carbonyl was invariant, i.e., in all cases trans to the Asp/Glu protein-based ligand, in the KDM4A structure the dimethylamino group was observed to complex trans to His276 or trans to His188 in the two different molecules present in the asymmetric unit. Although in the cases of PHF8 and FIH the daminozide dimethylamino group was only observed to bind trans to the histidine equivalent to His276 of KDM4A, previous work[25] on complexes of 2OG oxygenases with 2OG and the cosubstrate analogue **12** demonstrates that the 1-carboxylate can bind in either of the two conformational positions, in a manner analogous to that observed for the dimethylamino group in the KDM4A structure. We propose that the selectivity of daminozide for the KDM2/7 subfamily could, at least in part, arise from a "snug fit" obtained via binding in the position trans to His247 wherein its two methyl groups are accommodated in a tight hydrophobic pocket (formed by Val255, Ile313, and Tyr257), which is conserved in the KDM2/7subfamily (Figure 4). This pocket might bind the daminozide methyl groups less tightly in other demethylases/oxygenases because it is more hydrophilic (as demonstrated by crystallographic analysis) or

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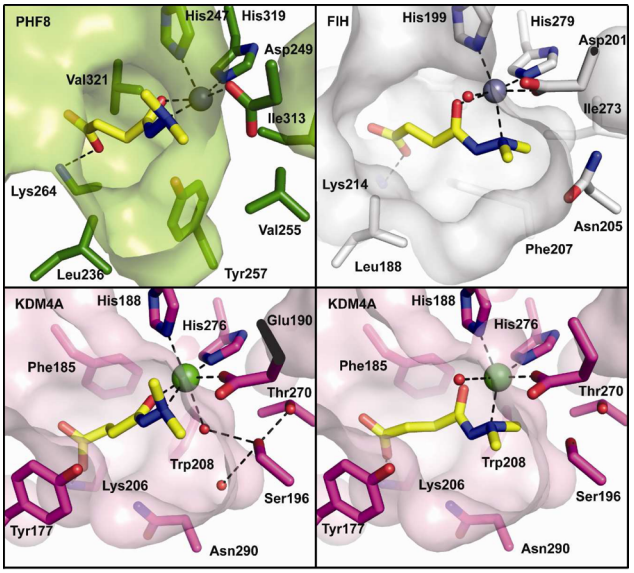

Figure 4. Crystal structures reveal the mode of inhibition of the KDM2/7 subfamily by daminozide: PHF8 (with Zn(II) substituting for Fe(II)) and KDM4A (with Ni(II) substituting for Fe(II)) and FIH (with Zn(II) substituting for Fe(II)) (Table S3). Daminozide binds the metal through its hydrazide amine lone pair and carbonyl oxygen. Two distinct orientations of daminozide are observed in the two KDM4A molecules in the asymmetric unit; both are shown (see Table S2 for electron density maps). Selectivity of daminozide for the KDM2/7 subfamily may arise because they possess a hydrophobic region (Tyr257, Val255, and Ile313) into which the two daminozide methyl groups bind. In contrast, the equivalent regions of KDM4A, FIH, and the other tested demethylases/oxygenases are more hydrophilic.

predicted to be more hydrophilic (by structure based on sequence alignments) for all the other 2OG oxygenases tested (Figure 1) (e.g., for KDM4A, they are Ser196, Thr270, and Asn198; for KDM6B, they are Ser225, Ile291, and Asn227; for FIH, they are Asn205, Ile273, and Phe257).

We also synthesized and tested analogues of daminozide (Table 1 and Table S3). The trimethylated analogue **22** and **27**, both of which lack the terminal amine lone pair, displayed little/no KDM inhibition, consistent with the proposed mode of daminozide inhibition involving chelation by its terminal hydrazide amine. The monomethylated **23** and unmethylated analogues **24** were more potent than daminozide against KDM2A. However, when tested against other 2OG subfamilies, these compounds were substantially less selective than daminozide (and were somewhat unstable in aqueous solution). Succinylhydroxamic acid **25** and dioxoheptanoic acid **28**, in which the acylhydrazinamide of daminozide is replaced by metal-chelating hydroxamic acid and malonyl groups, respectively, were also relatively potent but nonselective inhibitors (Table 1). Notably, **26**, in which the amide nitrogen of daminozide is N-methylated, is also selective for the KDM2/7demethylases, albeit with reduced potency. Like daminozide, **26** has two methyl groups on the acylhydrazide amine, suggesting that their presence confers selectivity. The other tested analogues were less active (Table S3).

CONCLUSIONS

Overall, we have demonstrated how the development of a screening platform employing multiple human 2OG oxygenases can enable the discovery of compounds selective for particular subfamilies. In the long term, we aim to help develop screens for all human 2OG oxygenases and see them applied to the discovery of medicinally useful inhibitors. By use of a relatively focused set of 2OG analogues, the screening platform led to the discovery that daminozide is a selective inhibitor of the KDM2/7demethylase subfamily. While daminozide itself is unlikely to be of medicinal use, the work has revealed a new class of 2OG oxygenase inhibitor, which employs alkylamino iron chelation. Of particular note is the high degree of selectivity exhibited by daminozide, especially given its achiral nature and low molecular weight. The precise mode of action of daminozide as a plant growth regulator[16,17] and its potential human toxicity (if any) under physiologically relevant conditions are unknown. We have no evidence that our results are directly relevant to the toxicity issues relating to daminozide. However, the results do suggest a potential of daminozide or its derivatives to exert biological effects via the inhibition of 2OG oxygenases involved in epigenetics in animals and, potentially, in other organisms including plants. Given the link between JmjC enzymes and diseases including cancer,[2,3] further work on the biological effects of daminozide is of interest.

ASSOCIATED CONTENT

Supporting Information

Protein expression and purification methods, assay methods, synthetic procedures, characterization of all synthesized compounds, ^{1}H NMR spectra of daminozide and analogues with iron(II), and crystalllographic data collection and refinement statistics. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

PDB codes: 4AI9, 4AI8, 4DO0 for complex of daminozide with KDM4A, FIH, PHF8 proteins, respectively.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

2OG, 2-oxoglutarate; BBOX, γ-butyrobetaine hydroxylase; FBXL, F-box, leucine-rich repeat protein; FIH, factor inhibiting hypoxia inducible factor; HDAC, histone deacetylase; JARID, jumonji, AT rich interactive domain containing protein; JmjC, jumonji-C domain; JMJD, jumonji-C domain containing protein; PHD2, human prolyl hydroxylase; PHF, plant homeodomain containing protein

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