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Synthesis and activity of *N*-oxalylglycine and its derivatives as Jumonji C-domain-containing histone lysine demethylase inhibitors

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ABSTRACT

N-Oxalylglycine (NOG) derivatives were synthesized, and their inhibitory effect on histone lysine demethylase activity was evaluated. NOG and compound **1** inhibited histone lysine demethylases JMJD2A, 2C and 2D in enzyme assays, and their dimethyl ester prodrugs DMOG and **21** exerted histone lysine methylating activity in cellular assays.

Methylation of lysine residues in the N-terminal region of the core histones plays a pivotal role in the regulation of gene expression.[1] It has been reported that methylation of histone lysine residues occurs on lysine 26 of histone 1 (H1K26), H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20.[2] Methylated lysine can be in a mono-, di-, or tri-methylated form, and these different forms differentially affect chromatin structure and are responsible for transcriptional activation as well as silencing.[3]

In contrast to other histone modifications such as acetylation and phosphorylation, histone methylation had been regarded as irreversible because of the high thermodynamic stability of the N–C bond. However, two classes of histone lysine demethylases have recently been identified. One is lysine-specific demethylase 1, a flavin-dependent amine oxidase domain-containing enzyme.[4] The other comprises the Jumonji C domain-containing histone demethylases (JHDMs) such as JMJD2A, 2B, 2C and 2D.[5] JHDMs are Fe(II) and α-ketoglutarate dependent enzymes that oxygenate methylated histone lysine residues and thereby cause their demethylation after releasing formaldehyde, carbon dioxide and succinic acid. The identification of these enzymes established that histone methylation is reversibly regulated by histone lysine methyltransferases and histone lysine demethylases.[6]

JHDMs are implicated in cancer cell growth.[2] For example, it was shown that JMJD2C is associated with the growth of oesophagal squamous cancer, and JMJD2A, 2B and 2C are involved in prostate cancer.[5,7] Therefore, JHDM inhibitors can be not only tools to study the function of these enzymes, but also serve as potential anticancer agents.

To date, only a few JHDM inhibitors have been identified (Fig. 1). *N*-Oxalylglycine (NOG), the amide analogue of α-ketoglutarate, has been reported to inhibit JMJD2C in an in vitro assay,[7] but has not been tested in a cellular assay. Succinic acid has been suggested to inhibit JMJD2D by product inhibition.[8] In addition, during the course of our study presented in this report, 2,4-lutidinic acid, which is known as an inhibitor of other Fe(II)/α-ketoglutarate dependent oxygenases, was reported as an inhibitor of JMJD2A and 2E,[9] although it was also not examined in a cellular assay. To the best of our knowledge, there is no report on selective JHDM inhibitors. Therefore, we initiated a search for novel JHDM inhibitors with the goal of drug discovery as well as finding new tools for biological research. In this letter, we describe the design, synthesis, JHDM inhibition activity and cellular activity of NOG and its derivatives.

We designed JHDM inhibitors based on the crystal structure of JMJD2A complexed with NOG and histone trimethylated lysine

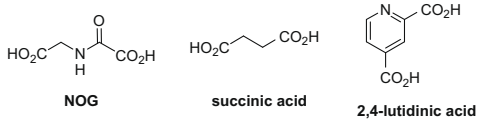

Figure 1. Structures of previously reported JHDM inhibitors.

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peptide (PDB ID 2OQ6).[10] The crystal structure showed that the oxalyl group of NOG interacts with Fe(II), and the other carboxyl group forms a hydrogen bond with Tyr 132 in the active centre of JMJD2A (Fig. 2, left). In addition, the trimethylamino group of histone trimethylated lysine peptide is surrounded by Gly 170, Tyr 175, Glu 190 and Ser 288. On the basis of this structure, we designed potential selective JHDM inhibitors **1**–**4** (Fig. 3) in which NOG is connected with a dimethylamino group through a linker (Fig. 2, right). We anticipated that the nitrogen atom of the dimethylamino group forms a hydrogen bond with the hydroxyl group of Tyr 175 or Ser 288,[11,12] and the aryl linker interacts with the aromatic rings of Tyr 177, Phe 185 and Trp 208, which might lead to the selective inhibition of JHDMs.

The synthesis of compound **1** is outlined in Scheme 1. The primary amine of 4-dimethylaminobenzylamine **5** was alkylated with *tert*-butyl bromoacetate to give secondary amine **6**. Treatment of compound **6** with *tert*-butyl chloroglyoxylate afforded tertiary amine **7**. Removal of the two *tert*-butyl groups of **7** under acidic conditions gave the desired dicarboxylic acid **1**.

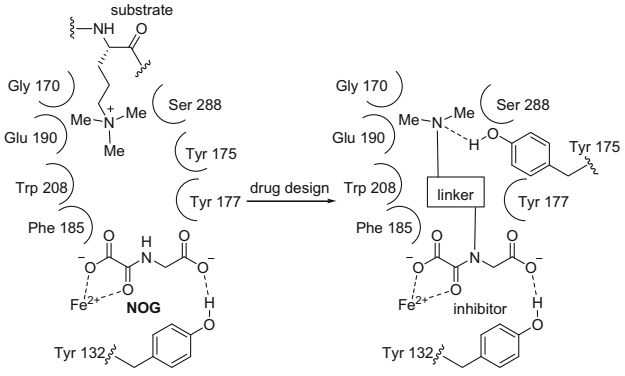


Figure 2. Interaction of NOG and trimethylated lysine substrate with JMJD2A (left), and models for the binding of designed inhibitors (right).

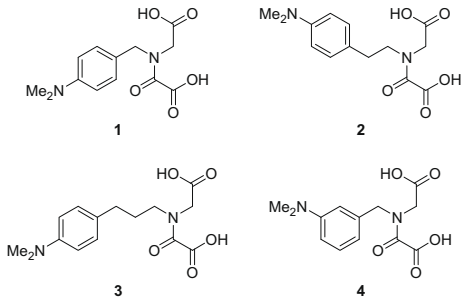
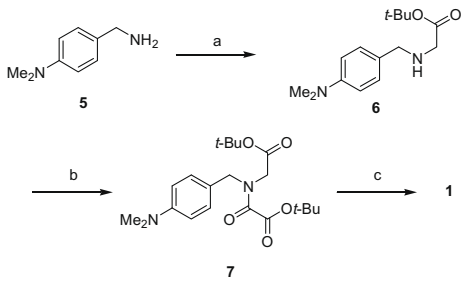
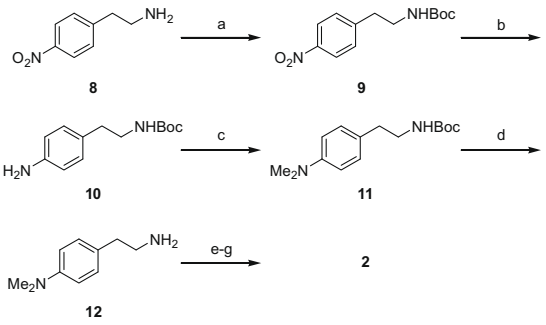


Figure 3. Structures of compounds **1**–**4**.



Scheme 1. Reagents and conditions: (a) *tert*-butyl bromoacetate, Et_3N, CH_2Cl_2, rt, 54%; (b) *tert*-butyl chloroglyoxylate, Et_3N, CH_2Cl_2, 0 °C, 58%; (c) HCl, AcOEt, CH_2Cl_2, 0 °C to rt, 59%.

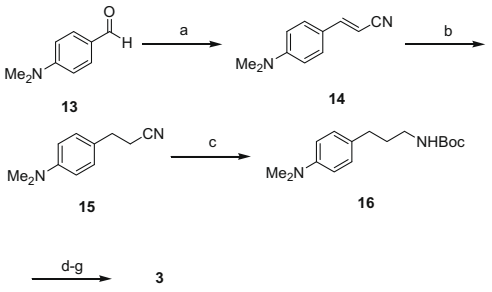


Scheme 2. Reagents and conditions: (a) $(Boc)_2O$, Et_3N, THF, 0 °C to rt, 82%; (b) H_2, Pd/C, EtOH, rt, 90%; (c) MeI, K_2CO_3, DMF, rt, 34%; (d) HCl, AcOEt, CH_2Cl_2, 0 °C to rt, 94%; (e) *tert*-butyl bromoacetate, Et_3N, CH_2Cl_2, rt; (f) *tert*-butyl chloroglyoxylate, Et_3N, CH_2Cl_2, rt; (g) HCl, AcOEt, $CHCl_3$, 0 °C to rt, 26% (three steps).

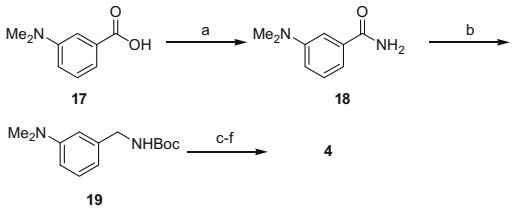
Scheme 2 shows the preparation of compound **2**. Reaction of 4-nitrophenethylamine **8** with $(Boc)_2O$ gave Boc-protected compound **9**. Reduction of the nitro group of **9** gave aniline **10**. Aniline **10** was reacted with iodomethane to give dimethylamine **11**. Then, deprotection of **11** using hydrochloric acid yielded amine **12**. Compound **2** was prepared from amine **12** using the procedure described for the synthesis of **1**.

Preparation of compound **3** is shown in Scheme 3. Horner–Wadsworth–Emmons reaction was applied to the conversion of benzaldehyde **13** into cinnamonitrile **14**. The double bond of **14** was hydrogenated to give compound **15**. The cyano group of **15** was then reduced, and subsequent treatment with $(Boc)_2O$ gave compound **16**. *N*-Boc compound **16** was converted to compound **3** using the procedure described for the synthesis of **1** and **2**.

Scheme 4 illustrates the synthesis of compound **4**. Coupling between carboxylic acid **17** and NH_3 in the presence of EDCI and



Scheme 3. Reagents and conditions: (a) cyanomethylphosphoic acid diethyl ester, NaH, THF, 0 °C, 66%; (b) H_2, Pd/C, MeOH, 83%; (c) (i) BH_3?SMe_2, THF, reflux; (ii) $(Boc)_2O$, Et_3N, THF, rt, 58%; (d) HCl, AcOEt, CH_2Cl_2, 0 °C to rt, 80%; (e) *tert*-butyl bromoacetate, Et_3N, CH_2Cl_2, rt; (f) *tert*-butyl chloroglyoxylate, Et_3N, CH_2Cl_2, 0 °C, rt; (g) HCl, AcOEt, CH_2Cl_2, 0 °C to rt, 12% (three steps).



Scheme 4. Reagents and conditions: (a) NH_3, EDCI, $HOBt{\cdot}H_2O$, DMF, H_2O, rt, 54%; (b) (i) $LiAlH_4$, THF, reflux; (ii) $(Boc)_2O$, Et_3N, THF, rt, 87%; (c) HCl, AcOEt, 0 °C to rt; (d) *tert*-butyl bromoacetate, Et_3N, CH_2Cl_2, rt; (e) *tert*-butyl chloroglyoxylate, Et_3N, CH_2Cl_2, 0 °C, rt; (f) HCl, AcOEt, CH_2Cl_2, 0 °C to rt, 23% (four steps).

HOBt afforded amide **18**. The reduction of the amide group of **18**, followed by the treatment with $(Boc)_2O$ gave compound **19**. Compound **4** was prepared from compound **19** using the procedure described for the synthesis of **1** and **2**.

For the evaluation of the enzyme inhibitory activity of NOG and its derivatives **1**–**4**, we initially generated GST fusion proteins of the catalytic N-terminus of JMJD2A and 2C as well as of full-length JMJD2D.[13–16] Because JMJD2A, 2C and 2D have been reported to demethylate $H3K9me_3$,[14] we confirmed the histone demethylating activity of these three proteins in an in vitro assay and revealed demethylation with an antibody recognizing trimethylated H3K9 ($H3K9me_3$).[17–20] As shown in Figure 4, all three GST-JMJD2 fusion proteins completely removed $H3K9me_3$, whereas no demethylating activity was observed with mutated GST fusion proteins[13] in which homologous histidine residues in the catalytic centre were mutated to alanine. Using this system, we next assessed the impact of NOG and putative JMJD2 inhibitors **1**–**4** on the in vitro demethylation activity of GST-JMJD2 fusion proteins.[17] We first utilized inhibitors at 1 mM concentration and observed that only NOG significantly curtailed the demethylation activity of JMJD2A and JMJD2C (Fig. 5, lane 4). However, at a higher inhibitor concentration of 3 mM, significant inhibitory effects were also noted with compounds **1**, **2**, **4** and weakest with **3**. In contrast, JMJD2D enzymatic activity was not only strongly inhibited by 1 mM NOG, but also by 1 mM compounds **1**, **2** and **3**. Furthermore, compound **4** also displayed an inhibitory effect at 3 mM concentration. Thus, all compounds tested were able to inhibit the demethylation activity of JMJD2 proteins.

Next, we assessed if the inhibitors would display activity in cells.[14,21–25] We focused on the strongest inhibitors, NOG and compound **1**, and prepared dimethylester prodrugs of these compounds to allow for uptake through the cell membrane. Scheme 5 shows the synthesis of compound **21**, the dimethylester prodrug of compound **1**. Compound **5** was allowed to react with methyl bromoacetate to give compound **20**. Then, compound **20** was reacted with methyl chloroglyoxylate to give compound **21**.

In this cellular assay, the accumulation of $H3K9me_3$ and $H3K36me_3$ was examined, because JMJD2C has been reported to demethylate both H3K9 and H3K36.[14] As shown in Figure 6, overexpression of JMJD2C resulted in robust demethylation of $H3K9me_3$ and $H3K36me_3$ (compare lanes 1 and 5). Further, neither 100 mM dimethyl succinate (DMS), a previously reported JMJD2D inhibitor,[8] nor 2.5 mM compound **21** affected $H3K9me_3$ and $H3K36me_3$ in the presence of JMJD2C. However, 2.5 mM dimethylester of NOG (DMOG) resulted in enhanced $H3K9me_3$ and $H3K36me_3$ levels both in the presence of JMJD2C and in the absence of overexpressed JMJD2C, indicating that DMOG represses the demethylation activity of JHDMs including JMJD2C in cells. On the other hand, compound **21** slightly enhanced $H3K9me_3$ lev-

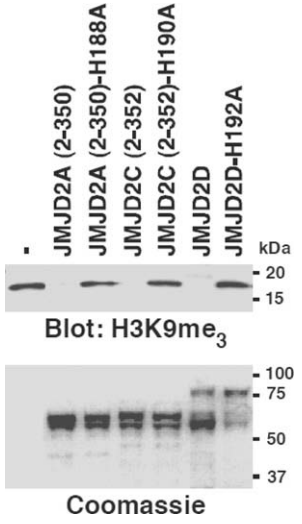


Figure 4. Demethylation ability of GST-JMJD2 fusion proteins (top) and Coomassie stained gel (bottom) indicating that comparable amounts of GST-JMJD2 fusion proteins were utilized.

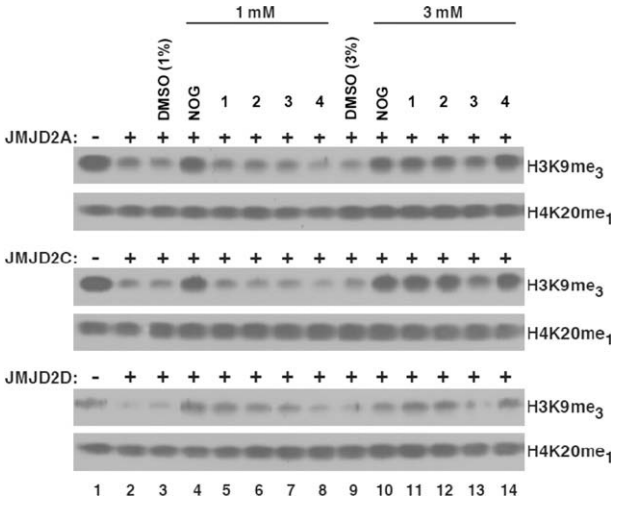
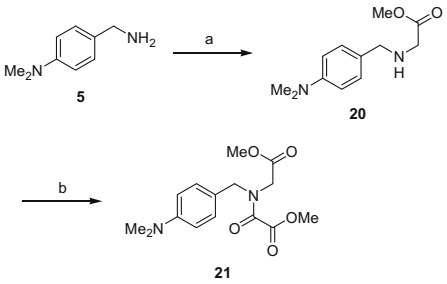


Figure 5. In vitro JMJD2 inhibition assay of NOG and compounds **1**–**4**. $H4K20me_1$ was assessed as a control and revealed to be unchanged.



Scheme 5. Reagents and conditions: (a) bromoacetic acid methyl ester, Et_3N, CH_2Cl_2, rt, 58%; (b) chloroglyoxylic acid methyl ester, Et_3N, CH_2Cl_2, 0 °C, 35%.

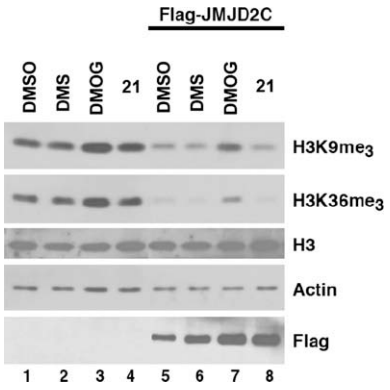


Figure 6. Demethylation in cells. 293T cells transfected with or without Flag-JMJD2C were treated with 100 mM dimethyl succinate (DMS), 2.5 mM oxalylglycine dimethylester (DMOG), 2.5 mM compound **21** or DMSO as a control. Demethylation activity of JMJD2C was assessed by anti-$H3K9me_3$ and anti-$H3K36me_3$ blotting. As loading controls, total H3 and actin levels were assessed, and expression of JMJD2C revealed by anti-Flag blotting.

els in the absence of overexpressed JMJD2C as compared with DMSO and DMS, suggesting that 2.5 mM compound **21** selectively inhibited the demethylase activity of a JHDM(s) other than JMJD2C in cells.

In summary, we have designed and synthesized NOG derivatives **1**–**4**[26] and evaluated the JMJD2-inhibitory activity of NOG and compounds **1**–**4** both in enzyme assays and in cellular assays that have been established for this study. Our data suggest that NOG and compound **1** show inhibitory activity against JHDMs and are more potent than succinic acid. As far as we could determine, this is the first report demonstrating that NOG and its derivative inhibit JHDMs both in enzyme assays and in cellular assays. The findings presented here should be valuable for further explorative studies uncovering more potent and selective JHDM inhibitors. Further investigations pertaining to NOG derivatives are progressing and will be reported in due course.

Acknowledgement

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13. Glutathione *S*-transferase (GST) fusion proteins of amino acids 2–350 of JMJD2A or its H188A mutant, of amino acids 2–352 of JMJD2C or its H190A mutant and of JMJD2D or its H192A mutant (Ref. 14) were produced in *Escherichia coli* and purified on glutathione-agarose beads (Ref. 15). Before freezing in liquid nitrogen, GST fusion proteins were dialyzed against 10 mM Hepes pH 7.4, 50 mM NaCl, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride and 1 mM DTT (Ref. 16).
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17. Reactions were set up in 18 μl of 50 mM Hepes pH 7.4, 50 mM NaCl, 50 mM KCl, 2 mM ascorbate, 0.2 mM $(NH_4)_2Fe(SO_4)_2$ and either 1 mM (Fig. 4) or 10 μM (Fig. 5) α-ketoglutarate employing 3 μg histones (USB) and 0.5 μg of indicated GST fusion proteins (Ref. 8). Where indicated, 1.8 μl or 5.4 μl of 10 mM inhibitor in 10% (v/v) DMSO or the vehicle DMSO itself was included in the reaction mixture. Reactions proceeded at 37 °C for 3 h (Fig. 4) or 1 h (Fig. 5, GST-JMJD2A and GST-JMJD2C) or 2 h (Fig. 5, GST-JMJD2D). After adding 18 μl of 2× Laemmli sample buffer and boiling for 10 min, supernatants were employed for SDS–PAGE (Ref. 18). Western blotting was then performed as described Ref. 19 utilizing antibodies from Upstate against H3K9me$_3$ (07-442; 1:4000 dilution) or H4K20me$_1$ (07-440; 1:2000 dilution). After incubation with secondary antibody coupled to horseradish peroxidase, enhanced chemiluminescence was employed to reveal proteins (Ref. 20).
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21. 293T cells were seeded in poly-L-Lysine coated 12 wells and grown to 25% confluency. Then, cells were transiently transfected by the calcium phosphate coprecipitation method essentially as described (Refs. 22,23). 1.7 μg of pBluescript KS$^+$ and 0.5 μg of empty vector pEV3S or Flag-JMJD2C expression vector (Ref. 24) were utilized for transfection. 12 h after addition of the precipitate to the cells, it was removed by washing twice with phosphate-buffered saline (Ref. 25). Cells were then incubated in 600 μl DMEM + 10% fetal calf serum for 12 h in a humidified atmosphere containing 10% CO_2. Then, medium was replaced with fresh one containing 1% of DMSO or inhibitors dissolved in DMSO and cells were incubated for another 24 h. Cells were then lysed in 80 μl of 10 mM Tris, 30 mM $Na_4P_2O_7$, 200 mM NaCl, 50 mM NaF, 2 mM DTT, 1% triton X-100 (pH 7.1) supplemented with 20 μg/ml leupeptin, 2 μg/ml pepstatin A, 4 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. After 45 min on ice, an equal volume of 2× Laemmli sample buffer was added, the extracts boiled for 12 min, debris removed by centrifugation and supernatants employed for SDS–PAGE and Western blotting as described above. The following antibodies at the indicated dilutions were used: H3K9me$_3$ (1:4000; 07-442, Upstate), H3K36me$_3$ (1:4000; Ab9050, Abcam), H3 (1:500; sc-10809, Santa Cruz), Actin (1:500; A2066, Sigma) and Flag (1:5000; M2, Sigma).
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26. The purity of compounds **1**–**4** and **21** was certified by elemental analysis. **1**·HCl: Anal. Calcd for $C_{13}H_{16}N_2O_5{\cdot}HCl{\cdot}1/4H_2O$: C, 48.60; H, 5.49; N, 8.72. Found: C, 48.89; H, 5.80; N, 8.43. **2**·HCl: Anal. Calcd for $C_{14}H_{18}N_2O_5{\cdot}HCl$: C, 50.84; H, 5.79; N, 8.47. Found: C, 50.56; H, 5.67; N, 8.48. **3**·HCl: Anal. Calcd for $C_{15}H_{21}N_2O_5{\cdot}HCl{\cdot}1/10H_2O$: C, 51.91; H, 6.17; N, 8.07. Found: C, 52.25; H, 6.14; N, 8.12. **4**·HCl: Anal. Calcd for $C_{13}H_{16}N_2O_5{\cdot}HCl$: C, 49.30; H, 5.41; N, 8.84. Found: C, 49.13; H, 5.59; N, 8.64. **21**: Anal. Calcd for $C_{15}H_{20}N_2O_5$: C, 58.43; H, 6.54; N, 9.09. Found: C, 58.35; H, 6.52; N, 9.05.