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Inhibition of Histone Demethylases by 4-Carboxy-2,2′-Bipyridyl Compounds

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In eukaryotes, nuclear DNA is packaged into chromatin by binding to histones and associated factors. Covalent modifications to histone tails are associated with specific transcriptional states of the associated DNA. Acetylation of lysine side-chains normally correlates with transcriptional activation, while deacetylation leads to transcriptional silencing. The regulatory roles of methylation of lysine and arginine residues appear to be more complex. Methylation of certain lysine residues is associated with active transcription, while methylation of others is associated with silencing and heterochromatin formation. Each methylation ‘mark’ is placed, removed and ‘interpreted’ in a site-specific manner by histone methyltransferases, demethylases and methyl-binding domains, respectively. The biological functions of the individual enzymes are largely undefined, and are the focus of current investigations (for review see [1, 2])

The JmjC histone demethylases are 2-oxoglutarate (2OG) dependent oxygenases that catalyse N^{ε}-lysyl demethylation via hydroxylation of the methyl group in a 2-oxoglutarate and Fe(II)-dependent manner (Scheme 1). Human 2OG oxygenases catalyse a range of reactions, including hydroxylation of amino acids, DNA and small molecules, and demethylation of proteins and DNA.[3] 2OG oxygenases show promise as therapeutic targets. An inhibitor of γ-butyrobetaine hydroxylase (BBOX) is used for the treatment of cardiovascular disease[4, 5] and inhibitors of the hypoxia inducible factor (HIF) prolyl hydroxylases are in clinical trials for the treatment of anaemia.[6] Inhibitors of the collagen prolyl hydroxylases have also been evaluated as potential therapeutics for the treatment of liver fibrosis.[7, 8] The discovery of the JmjC domain histone demethylases, and the suggestions that some of them are potential therapeutic targets for cancer treatment,[9] has

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stimulated interest in their inhibition, but relatively few studies have been described. Reported inhibitors of the JmjC demethylases include *N*-oxalyl amino acids, 8-hydroxyquinolines, pyridine dicarboxylates, hydroxamic acids and catechol-type flavonoids (Figure 1).[10-13] Compounds which catalyse the ejection of a structural Zn(II) ion from the JMJD2 demethylases have also been reported (Figure 1). [14]

In a study describing various template inhibitors of the JmjC demethylases, we found that 2,2′-bipyridyl compounds with at least one 4-carboxylate group inhibit the histone demethylase JMJD2E.[11] A related series of compounds, 5,5′-dicarboxylate-2,2′-bipyridyls, is reported to inhibit the collagen prolyl-4-hydroxylases.[15] 2,2′-Bipyridine and bipyridyl compounds have also been used as inhibitors of the HIF hydroxylases.[16] Although it is likely that in some cases the enzyme inhibition effects of bipyridyl compounds result from metal chelation in solution, they also have the potential to inhibit via active site binding, as is the case for some 2OG oxygenases; however, to date there is no structural information on their mechanism of action. Here we report structure-activity relationship studies and analyses on bipyridyl inhibitors of JMJD2E.

The bipyridyl compounds tested were synthesised according to Scheme 2. Thus, 4,4′-dicarboxy-2,2′-bipyridine **9** was esterified to give the dimethyl or diethyl esters, which were then hydrolysed to yield the mono-esters **8a** or **8b** respectively. **8a** was coupled to a set of suitably protected primary amines to yield compounds **11a-b**, **14a-e**, **16, 18a-b**, **20**, **22**, **24** and **27** which were then hydrolysed and deprotected to yield the free carboxylic acids **12a-b**, **13a-b**, **15a-e**, **17, 19a-b**, **21**, **23**, **25** and **28**, respectively (Table 1). A derivative of 4-carboxy-2,2′-bipyridine, **30**, was synthesised to evaluate the importance of a 4-carboxyl group (Scheme 2).

Inhibition assays for the histone demethylase JMJD2E were carried out using two complementary assay methods. Inhibition of histone demethylation was measured using a coupled enzyme assay, employing formaldehyde dehydrogenase (FDH) to convert formaldehyde to formate, with concomitant reduction of NAD^+ to NADH, which was monitored spectrophotometrically.[11, 17, 18] Compounds were screened in 8-point serial dilutions against 100 nM JMJD2E, generating dose-response curves and IC_{50} data (Table 1); Each compound was also screened in a similar dose-response manner against FDH only, using 1μM formaldehyde as a substrate (to ensure that inhibitory effects were due to inhibition of JMJD2E and not FDH). To evaluate whether compounds were inhibiting by binding to the active site (as opposed to, or in addition to, chelation of iron in solution), non-denaturing electrospray ionisation protein mass spectrometry (ESI MS) (Figure 2) was used to detect binding of compounds to the intact protein complex.[11] Although there are caveats on the use of mass spectrometry for screening binding strength,[17] the results of both assay methods correlated well (Table 1), with more potent compounds showing a stronger binding interaction with the protein complex by ESI MS.

Previous work on inhibitors of the JMJD2 demethylases demonstrated that the parent compound 2,4-pyridinedicarboxylate is a 2OG-competitive inhibitor. This compound was shown by crystallographic analyses (PDB ID 2VD7) to bind the active-site metal via the pyridine nitrogen and the 2-carboxylate, while the 4-carboxylate is positioned to interact

with the 2OG-binding residues Lys206 and Tyr132, effectively 'anchoring' the compound in the active site (see Figure 4).[11] The bipyridyl compounds were, by analogy, predicted to bind in a similar manner, with the pyridinyl nitrogens chelating the active site metal, and the 4-carboxylate positioned to interact with Lys206 and Tyr132. In support of this proposal, all the bipyridyls tested were relatively potent inhibitors of JMJD2E (IC_{50}s <30 μM), with the exception of that lacking the 4-carboxylate, compound **30**, where the IC_{50} was 86 μM. This compound was 300-fold less potent than the equivalent compound with the 4-acid moiety, **15d**.

The bipyridyl derivative resulting from coupling with ethylenediamine, **13a**, inhibits JMJD2E with IC_{50} = 180 nM, showing a 36-fold improvement of inhibition compared to 4′-(methoxycarbonyl)-2,2′-bipyridine-4-carboxylic acid (IC_{50} = 6.6 μM). Analysis of a model of JMJD2A in complex with **13a** suggested that this improvement might in part be due to formation of a salt-bridge between the amine of bipyridyl derivative **13a** and Asp135 at the active site. To investigate the importance of the amine, the inhibition of JMJD2E activity by compound **23**, with a hydrogen substituting for an amino group, was evaluated. Compound **23** was found to have an IC_{50} of 13 μM, representing 72-fold lower potency than **13a**. Similarly, compounds **25** and **26** were significantly less potent than **13a**, suggesting that the amine of **13a** is important in binding.

The optimal position of the amine was then investigated. **13b** (IC_{50} = 470 nM) exhibited a 2-fold potency decrease over **13a**, which could reflect a sub-optimal length between the bipyridine core and Asp135. Consistent with a preference for the positive charge at this position, the IC_{50} values of **12a** and **12b**, where the amine is neutralized by a Boc group, were elevated (with IC_{50} = 3.2 μM and IC_{50} = 5.3 μM for **12a** and **12b** respectively). Introduction of unfunctionalised aromatic amides (**15a** with IC_{50} = 1.1 μM, and **15b**, IC_{50} 1.0 μM, resulted in a decrease in potency relative to **13a**. The naphthalene derivative (compound **21**) showed increased inhibition relative to its phenyl analogue (**15a**), suggesting that hydrophobic or π-π interactions in the active site might play a role in binding.

Based on the possibility that a combination of aromatic ring and amine might give improved potency, anilines **19a** and **19b** were synthesized and evaluated. The IC_{50}s of **19a** and **19b** (690 nM and 17 μM respectively) showed no improvements relative to **13a**, but demonstrated the importance of the regiochemistry of the position of the aromatic amine. Compounds **15c**, **15d** and **15e** were synthesized with the aim of tuning the length between the bipyridine and phenyl group. Compounds **15c**, **15d** and **15e** (with IC_{50}s of 110 nM, 270 nM and 320 nM respectively) had similar activities to **13a**, demonstrating that, at least for the compounds analysed, the chain lengths longer than one methylene all conferred similar potencies. These compounds might form π-π interactions between their phenyl ring and Tyr175 which is close to Asp135 in the substrate binding site. However, the addition of a second benzene ring in compound **28** caused a 20-fold loss of potency relative to its analogue **15c**.

Although bipyridyl compounds are expected to chelate Fe(II) in solution, it is clear from the ESI-MS binding data that these compounds inhibit by binding to the active site. Further, the IC_{50}s for most compounds are 10-fold to 100-fold less than the concentration of Fe(II) used

in the assays (10 μM), also indicating that their inhibitory effects are not due predominantly to iron chelation in solution. Finally, the substantial differences (>100 fold) in IC_{50} values between compounds anticipated to chelate iron similarly in solution (e.g between compound with a 4-carboxylate (**12a-28**) and one without the 4-carboxylate (**30**)) implies that these compounds inhibit at least in part as a result of specific interactions in the active site. To confirm this proposal, we also determined IC_{50}s for compounds **13a**, **15c** and **15d** at 50 μM Fe(II). The results showed that IC_{50}s did not shift with the increase in Fe(II) concentration (data not shown), indicating that these compounds inhibit JMJD2E predominantly by mechanisms other than Fe(II) chelation in solution.

We then worked to obtain a crystal structure for JMJD2A in complex with one of the more potent bipyridyl inhibitors, **13a.** A structure was solved for JMJD2A in complex with zinc, nickel (substituting for iron) and **13a** to 2.0 Å resolution using a reported structure (PDB ID: 2OX0) as a search model (Figure 3). The structure reveals that **13a** binds the active site metal by bidentate chelation through both pyridinyl nitrogens, verifying the proposed overall mode of iron binding of bipyridyl compounds to JMJD2A and likely other 2OG oxygenases including the HIF hydroxylases. Notably, the carboxylate of **13a** is positioned to interact with Lys206 and Tyr132 in a manner analogous to that observed for 2OG, so rationalising the difference in potency resulting from addition of a C-4 carboxylate to one of the pyridinyl rings. The amide nitrogen of **13a** is positioned to form two hydrogen bonds with water molecules which in turn form hydrogen bonds to the phenol oxygen of Tyr177 and to the backbone carbonyl oxygen of Glu169, and an electrostatic interaction with Asp135 (2.7 Å). For other potent inhibitors such as **15c-e** it is likely that the lack of the salt bridge with Asp135, and the flexibility of the aliphatic chain, would allow the inhibitors to adopt conformations placing the aliphatic/aromatic sidechains in the substrate binding region of the active site. This general mode of binding would also explain the relatively similar potencies of compounds **15c-e**, if the sidechains are projecting into the solvent-accessible region of the substrate binding groove.

Comparison of the crystal structure of the JMJD2A.**13a** complex with structures of JMJD2A in complex with fragments of its histone H3K9 and K36 substrates (PDB IDs 2OQ6 and 2OS2) reveals that, although **13a** does not occupy the N^{ε}-methyllysine binding groove, it is likely to block binding of the peptide substrate by occupying part of the binding site for the peptide backbone.

Comparison of the binding mode of compound **13a** with crystal structures of other inhibitors in complex with JMJD2A (2,4-PDCA, 5-carboxy-8-hydroxyquinoline, *N*-oxalyl-D-(*O*-benzyl)tyrosine and *N*-oxalylglycine) reveals that the bipyridyl compound binds in the same plane as the other two aromatic inhibitors, 2,4-PDCA and 5-carboxy-8-hydroxyquinoline, occupying the two coordination sites opposite His276 and Glu190 (Figure 4).[11, 12, 17, 19, 20] In contrast, 2OG, *N*-oxalylglycine and *N*-oxalyl-D-(*O*-benzyl)tyrosine coordinate opposite Glu190 and His188. In the case of 2OG this presumably leaves the site opposite His276 available for the binding of molecular oxygen. Although the situation with iron may be different to that for nickel (used for crystallography), these observations suggest that compounds such as **13a**, 2,4-PDCA and 5-carboxy-8-hydroxyquinoline may inhibit JMJD2

demethylases not only by competition with 2OG, but also by occupation of the oxygen binding site.

Overall we have shown that modifications to the 4,4′-dicarboxy-2,2′-bipyridine template can result in substantial increases in potency against JMDJ2E (IC_{50} of 6.6 μM for the lead compound **8a**, while the most potent compound identified, **15c**, had an IC_{50} of 110 nM, which represents a 66-fold improvement in potency).[11] These increases in potency are, at least in part, likely mediated by both hydrophobic/π-π interactions and, in the case of some compounds (e.g. **13a**), by electrostatic interactions. It is possible that potency of the bipyridyl compounds can be increased further by using the crystal structure of **13a** in complex with JMJD2A, including by further derivatisation of the 4-carboxylate to occupy the N^{ε}-methyllysine side chain binding groove. Notably, bipyridyl compounds have been found to inhibit both the collagen prolyl-4-hydroxylases[15, 21] and the hypoxia inducible factor prolyl-4-hydroxylases,[16] suggesting that these compounds might be relatively generic inhibitors of 2OG oxygenases. Our work could thus enable the rational structure-based functionalisation of the bipyridyl template to enable the production of inhibitors with different selectivities for use in biological research and medicinal applications.

Experimental Section

Experimental details, including compound synthesis, inhibition assays, protein purification and crystallography, are contained in the electronic supporting information available online.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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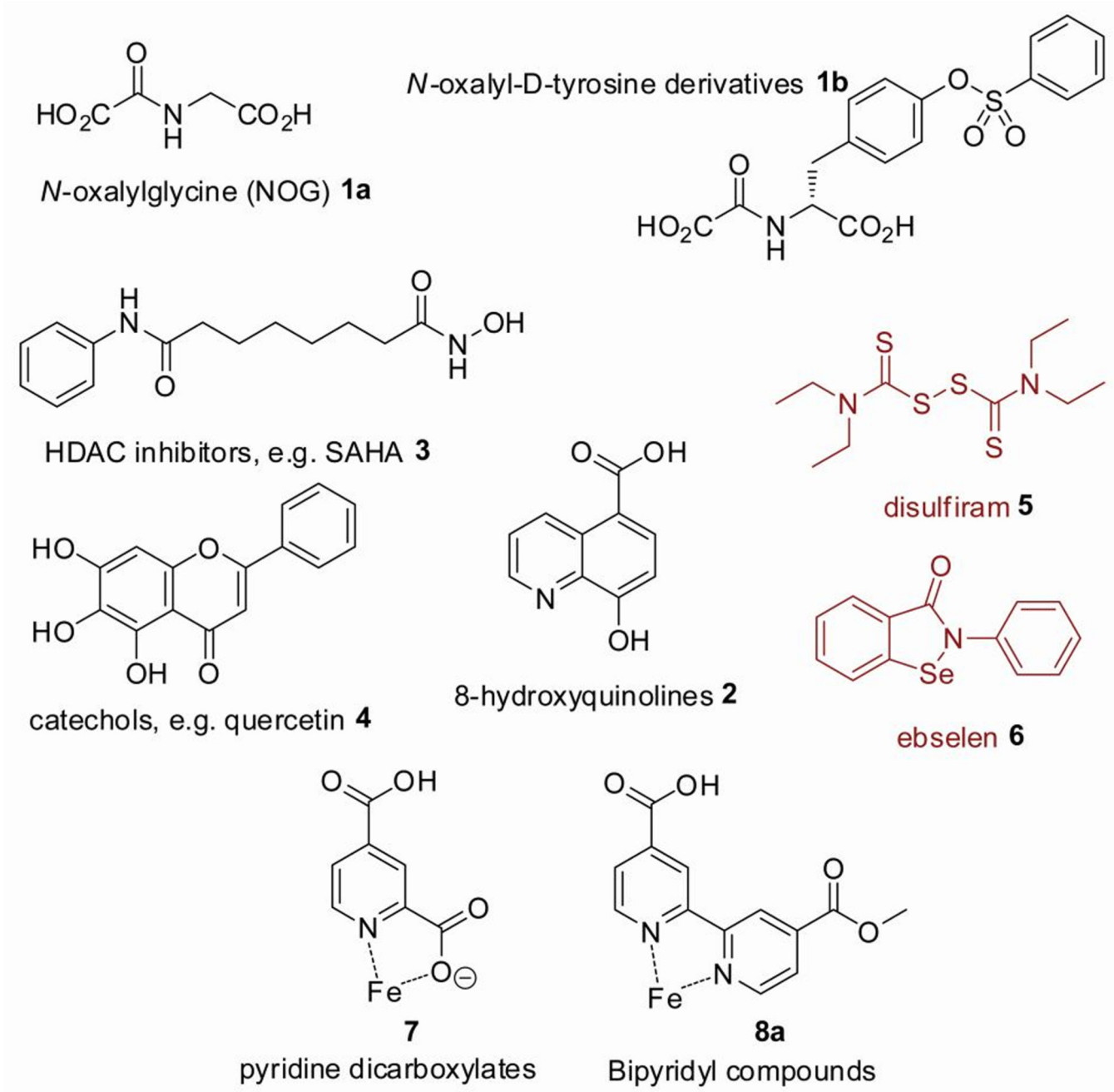

Figure 1.

Structures of some previously reported histone demethylase inhibitors. Compounds **5** and **6** inhibit by ejecting Zn(II) from the enzyme.

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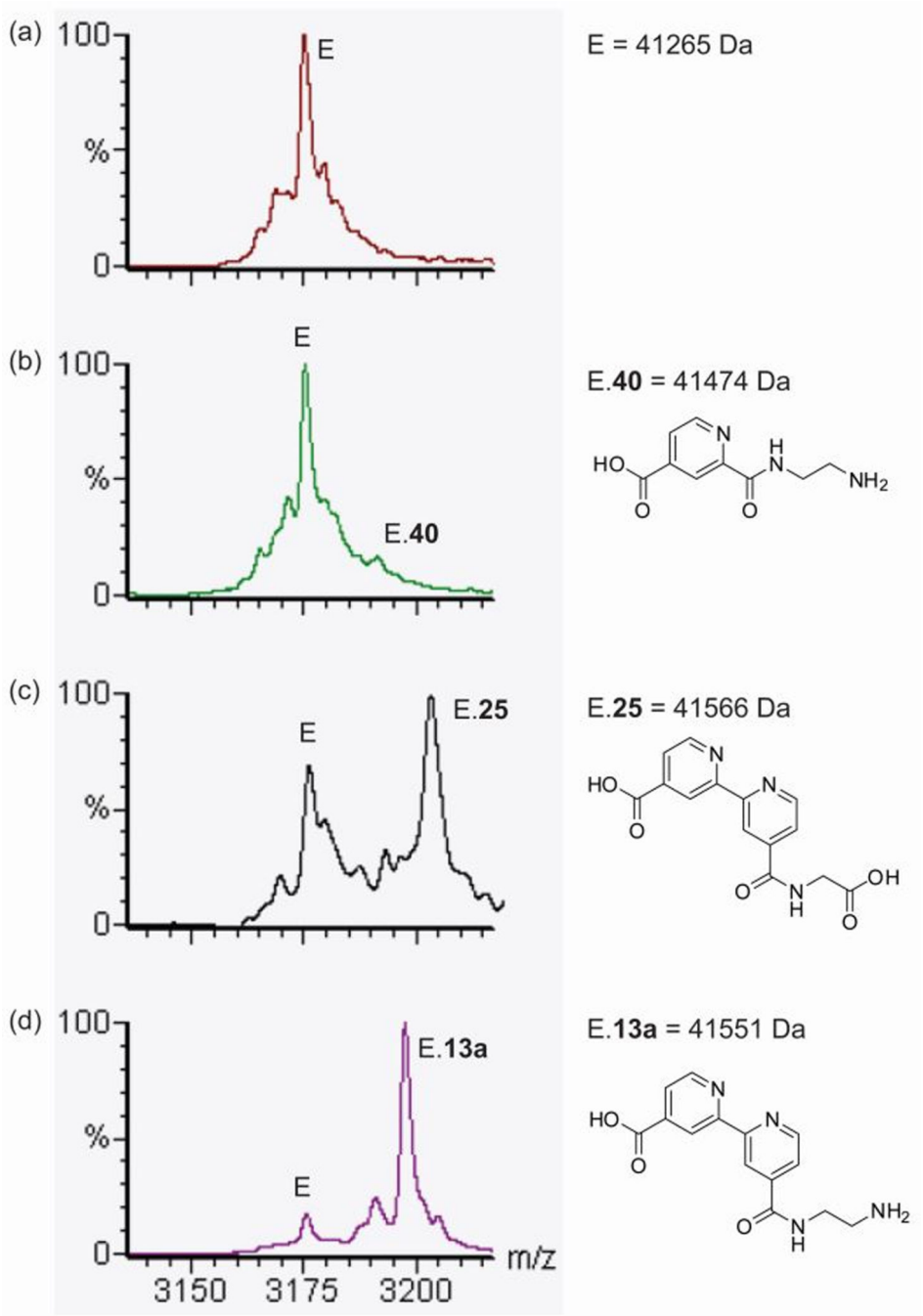

Figure 2.

Non-denaturing ESI-MS binding assay of inhibitors to JMJD2E. (a) JMJD2E.Fe(II) (labeled E) in the presence of compounds (b) **40**, (c) **25** and (d) **13a**, showing examples of relatively weak, medium and strong binding compounds, respectively.

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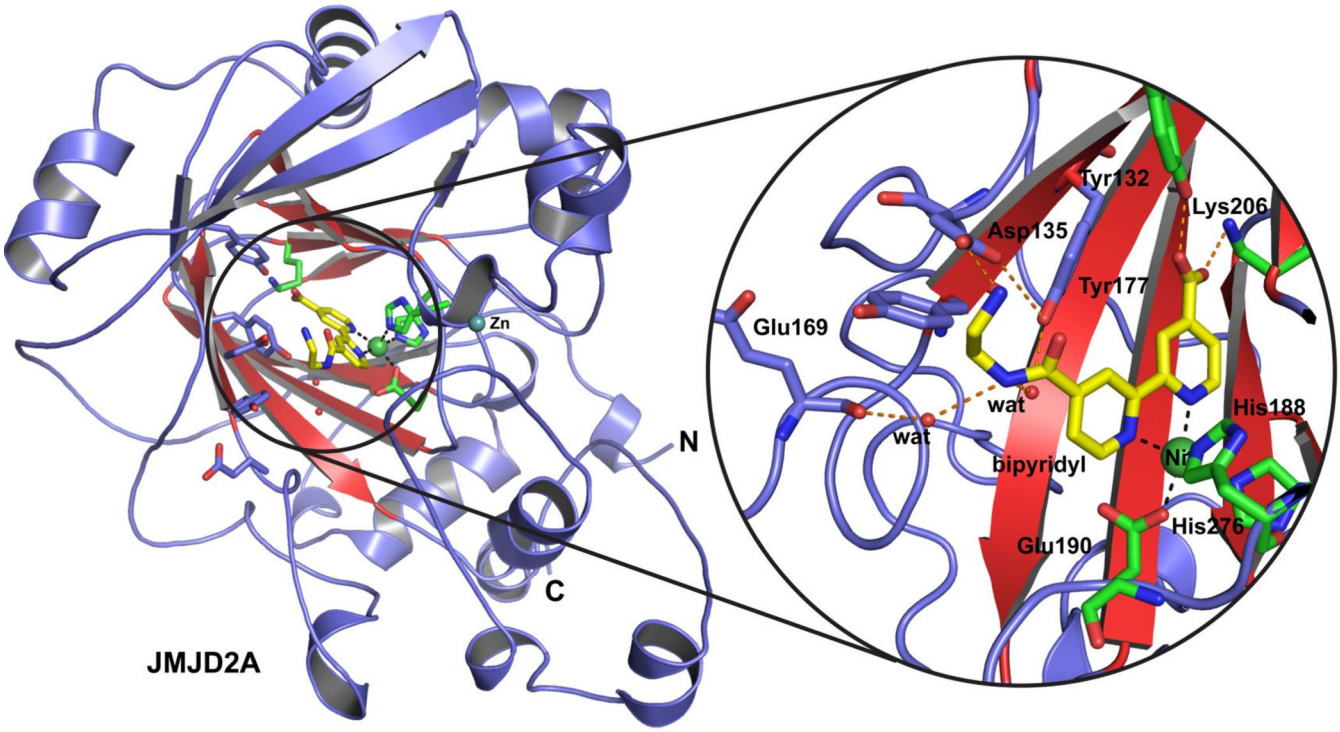


Figure 3.

Views from the crystal structure of JMJD2A in complex with compound **13a** (yellow sticks). The double-stranded β-helix (conserved in 2OG oxygenases) is in red. Residues that bind Fe(II) and 2OG are shown as green sticks, Ni(II), which replaces Fe(II) for crystallography, is shown as a green sphere. Other residues likely interacting with **13a** are shown as blue sticks.

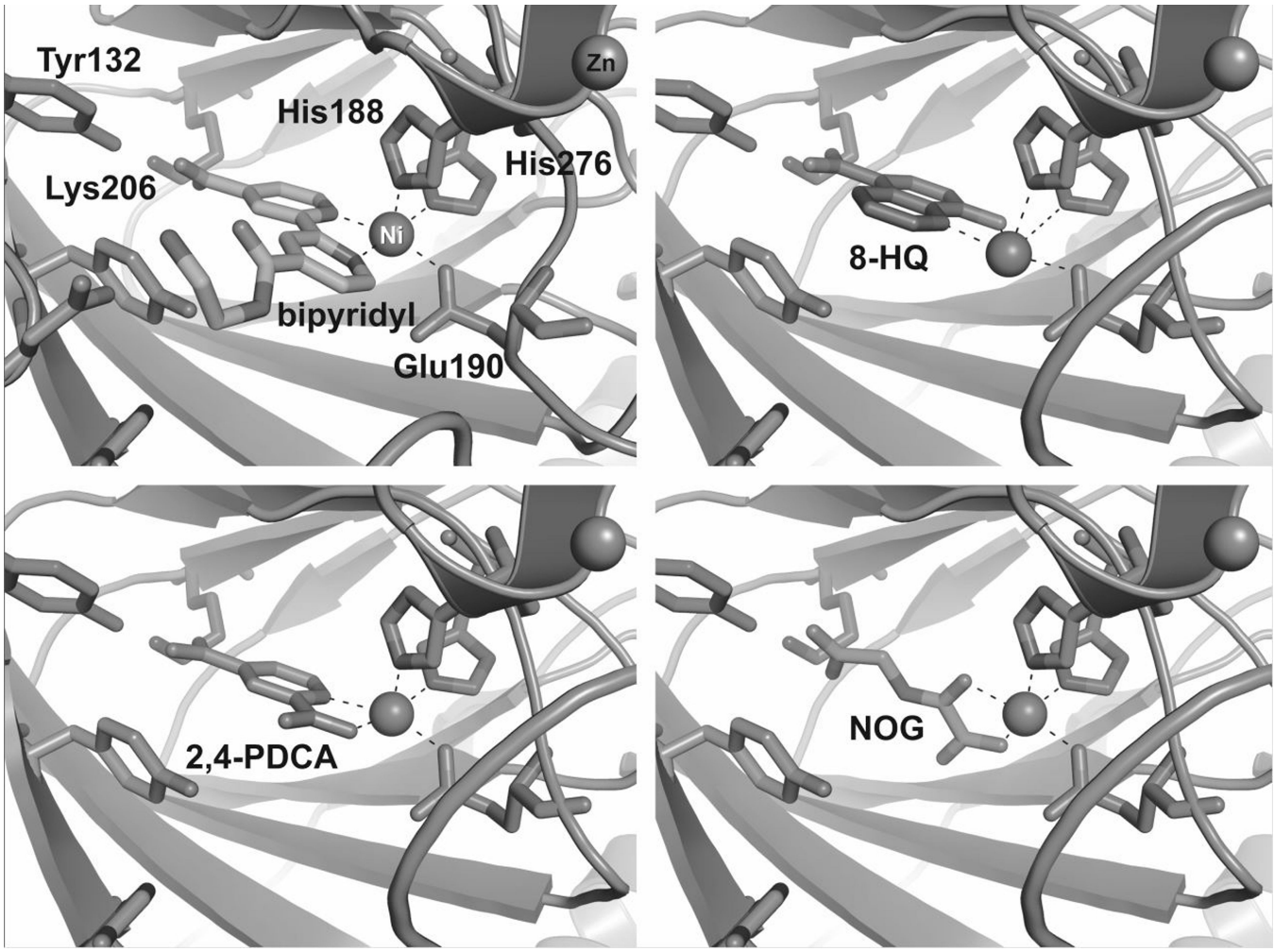
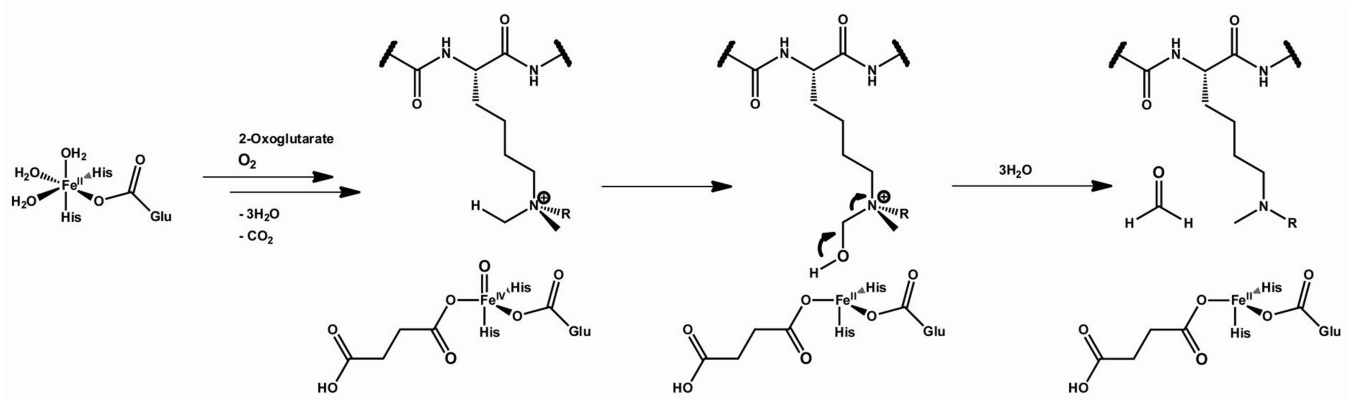


Figure 4.

Comparison of metal coordination by various JMJD2 demethylase inhibitors. The bipyridyl compound **13a**, 8-hydroxyquinoline (8HQ) and 2,4-pyridine-dicarboxylate inhibitors coordinate the active site metal (Ni(II) substitutes for Fe(II) for crystallography) by occupying the two coordination sites opposite His276 and Glu190. In contrast, *N*-oxalylglycine (NOG), a 2OG analogue, coordinates by occupying the coordination sites opposite Glu190 and His188.



Scheme 1.

The JmjC-domain histone demethylases catalyse *N*-demethylation. For each demethylation reaction, 2OG is oxidised by molecular oxygen to generate succinate, CO_2 and a reactive ferryl-oxo species which hydroxylates lysyl N^{ε}-methyl groups; the unstable hemiaminal intermediate then fragments to release formaldehyde and the demethylated lysine residue.

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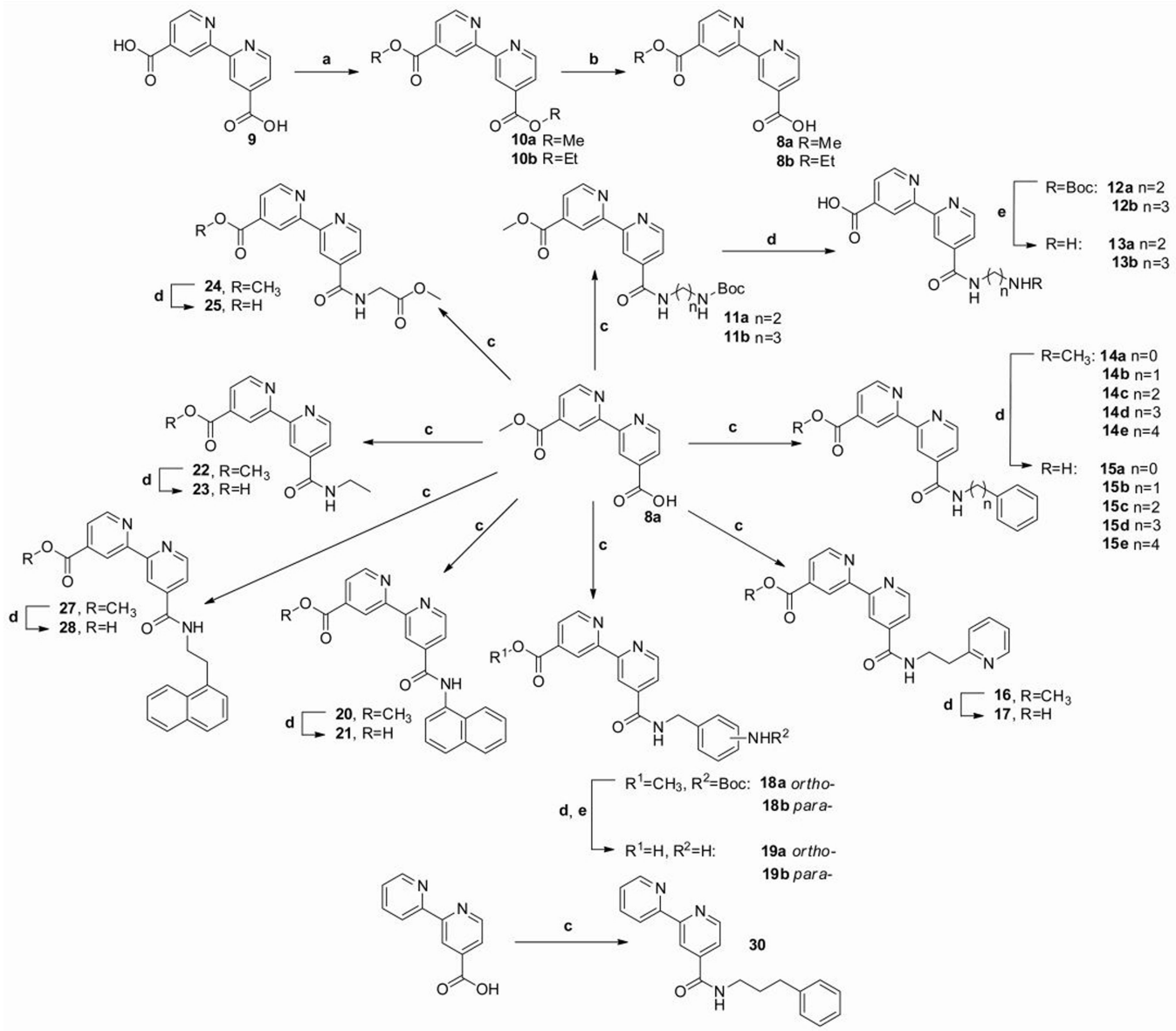

Scheme 2.

Reagents and conditions: a) $SOCl_2$, MeOH, reflux, overnight, 90 %; b) KOH, MeOH/THF (1:1), overnight, reflux, 70 %; c) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), 1-hydroxybenzotriazole (HOBT), triethylamine, DMF, overnight, r.t.; d) KOH, MeOH/THF, reflux, 4 h; e) CF_3COOH, 2% H_2O, 4h, r.t.

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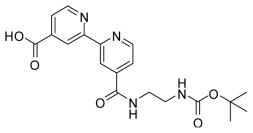
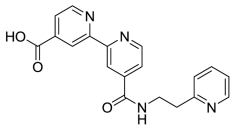
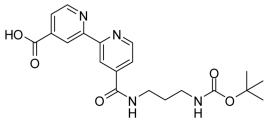
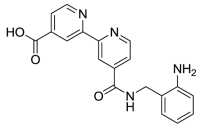
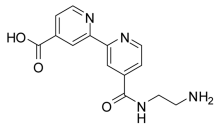
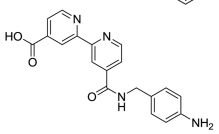
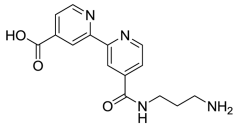
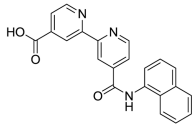
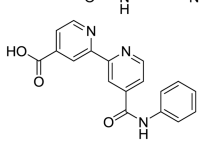
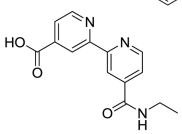
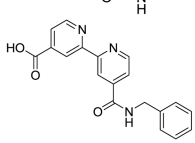
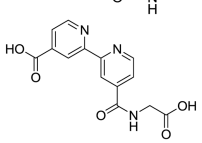
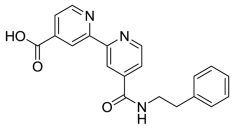
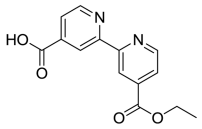
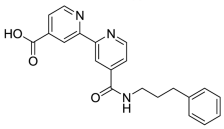
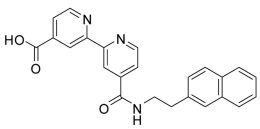
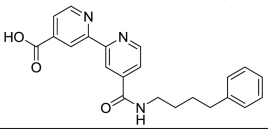
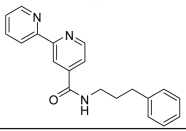
Table 1

Inhibition of the histone demethylase JMJD2E (100 nM) by bipyridyl compounds. IC_{50}s represent the result of three independent experiments, where standard errors in the log(IC_{50}) are less than 10 %. Ranking of binding strength by ESI-MS is as shown in Figure 3. All compounds were counter-screened against FDH at the same concentrations used for IC_{50} determinations, and inhibition of FDH was not observed under these conditions, implying that the compounds only inhibited JMJD2E.

Compound		IC_{50} (μM)	Ranking of binding (by ESI-MS)	Compound		IC_{50} (μM)	Ranking of binding (by ESI-MS)
12a		3.2	medium	**17**		1.1	strong
12b		5.3	medium	**19a**		0.69	strong
13a		0.18	strong	**19b**		17	strong
13b		0.47	strong	**21**		0.43	strong
15a		1.1	medium	**23**		13[b]	medium
15b		1.0	strong	**25**		25[b]	medium
15c		0.11	strong	**8b**		27[b]	medium
15d		0.27	strong	**28**		2.3	N.D.
15e		0.32	strong	**30**		86	N.D.