Chem Soc Rev

View Online
Dynamic Article Links

Cite this: DOI: 10.1039/c0cs00203h

www.rsc.org/csr

CRITICAL REVIEW

Inhibition of 2-oxoglutarate dependent oxygenases†

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Received 3rd December 2010
DOI: 10.1039/c0cs00203h

2-Oxoglutarate (2OG) dependent oxygenases are ubiquitous iron enzymes that couple substrate oxidation to the conversion of 2OG to succinate and carbon dioxide. In humans their roles include collagen biosynthesis, fatty acid metabolism, DNA repair, RNA and chromatin modifications, and hypoxic sensing. Commercial applications of 2OG oxygenase inhibitors began with plant growth retardants, and now extend to a clinically used pharmaceutical compound for cardioprotection. Several 2OG oxygenases are now being targeted for therapeutic intervention for diseases including anaemia, inflammation and cancer. In this *critical review*, we describe studies on the inhibition of 2OG oxygenases, focusing on small molecules, and discuss the potential of 2OG oxygenases as therapeutic targets (295 references).

1. Introduction and historical perspective on 2OG oxygenases

Oxygenases catalyse the introduction of oxygen atoms that are derived directly from dioxygen into their products. The first 2OG-dependent oxygenases acting on biomacromolecules to be identified were the collagen hydroxylases.[1,2] The collagen hydroxylases use ferrous iron as a cofactor and 2-oxoglutarate (2OG) as a cosubstrate in addition to dioxygen (for review, see ref. 3). Following the pioneering work on the collagen hydroxylases, the 2OG oxygenases have emerged as a very widely distributed family. In addition to being involved in biological processes ranging from antibiotic biosynthesis in prokaryotes to oxygen sensing in humans, 2OG oxygenases also catalyse a wide range of oxidative reactions, possibly the widest of any identified enzyme family.[4] Reactions that they

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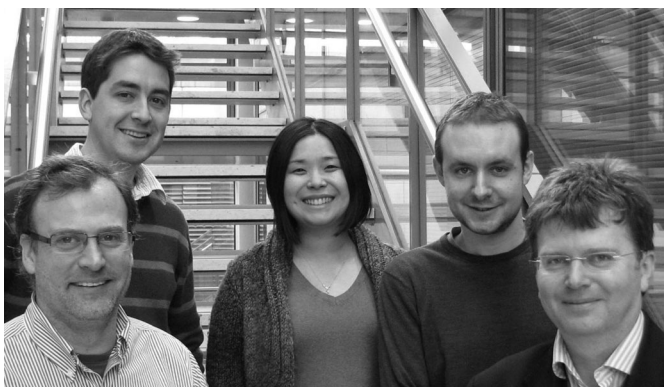
† Part of the themed issue on small molecules in biology.



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catalyse range from 'simple' hydroxylations and demethylations (*via* methyl group hydroxylation) to synthetically unprecedented ring closures and epimerisations.[4,5] Whole-genome sequencing efforts have revealed 2OG oxygenases as being present in most organisms, and have enabled functional assignment studies.[6,7] Structural studies on 2OG oxygenases have revealed a common protein fold and conserved cofactor and cosubstrate binding sites,[8] suggesting that 2OG oxygenases may be amenable to 'template-based' inhibitor design approaches.

In humans there are estimated to be ≥ 60 2OG oxygenase genes (Table 1).[7] Over the past decade new functional roles for human 2OG oxygenases have been discovered, including roles in biomedicinally important processes such as the hypoxic response, nucleic acid repair and modification, fatty acid metabolism, and chromatin modification.[6] These discoveries have opened up novel therapeutic possibilities including the targeting of human 2OG oxygenases by small molecules. These studies are at a relatively early stage compared to the 'family-based' efforts on some other protein families, such as kinases, G-protein coupled receptors (GPCRs), and proteases, but recent discoveries suggest that the 2OG oxygenases have significant therapeutic potential.

Initial studies on targeting human 2OG oxygenases began with the collagen prolyl hydroxylases for the treatment of fibrotic diseases.[9] Although these studies did not result in clinically useful inhibitors, the initial work highlighted the therapeutic potential of targeting 2OG oxygenases. Presently, 3-(2,2,2-trimethylhydrazine)propionate (THP), proposed to target a 2OG oxygenase involved in carnitine biosynthesis, is in clinical use as a cardioprotectant[10] in some countries and several small molecules targeting 2OG oxygenases are in clinical trials for the treatment of anaemia. Precedent that 2OG oxygenases can be targeted *in vivo* also comes from the long-standing commercial application of 2OG oxygenase inhibitors as plant growth retardants.[11] Here we review studies on small molecule inhibitors of 2OG oxygenases and discuss future prospects for their application, with particular focus on the human enzymes.

2. Structural and mechanistic considerations for 2OG oxygenases

Extensive structural and mechanistic information is now available for the 2OG oxygenases (reviewed in ref. 8 and 12). Pioneering crystallographic studies on 2OG oxygenases (and the closely related oxidase, isopenicillin N-synthase) involved in the biosynthesis of the β-lactam antibiotics (penicillins, cephalosporins and clavams),[13–15] and in cysteine metabolism (taurine dioxygenase)[16] led to the identification of a common fold and conserved active site elements for the 2OG oxygenases.[8] Although considerable gaps remain in our mechanistic knowledge, including a lack of detailed understanding of the conformational changes that occur during catalysis and the characterisation of some intermediates, the available structural information is sufficient to enable structure-based inhibitor design efforts. Table 2 lists crystal structures of 2OG oxygenases that have been reported in complex with inhibitors.

All identified 2OG oxygenases share a 'normally distorted' double-stranded β-helix (DSBH) fold, which is also known as a 'jelly-roll' or 'cupin' fold, as exemplified for a human prolyl hydroxylase in Fig. 1. The active site is positioned at the more open end of the 'barrel' formed by the eight strands of the DSBH fold.[8] Additional structural elements surrounding the DSBH core fold are always present in 2OG oxygenases, and include two highly conserved α-helices at its N-terminus and, often, a helix C-terminal to the DSBH. Because of the manner in which 2OG oxygenases probably fold, inserts of varying extent (*i.e.* from 10–30 or even up to 500 residues) between the fourth and fifth strands of the DSBH are sometimes present. This is exemplified by comparison of the asparaginyl hydroxylase Factor Inhibiting Hypoxia inducible factor (FIH), which has a long insert involved in substrate recognition, with the human prolyl hydroxylase PHD2, which has no β4–β5 insert (see Fig. 1).[12]

2OG oxygenases have been observed in various oligomeric states, but most commonly are monomeric or dimeric.[8,12] Dimerisation can involve N- and C-terminal elements, as observed for γ-butyrobetaine hydroxylase (BBOX)[17] which dimerises *via* its N-terminal Zn-binding domain, and FIH, which dimerises *via* two C-terminal helices, respectively.[18] Some 2OG oxygenases have more complex oligomerisation states, *e.g.* the collagen *trans*-4-prolyl-hydroxylases which exist as $\alpha_2\beta_2$-heterotetramers, in which the β-subunit is protein disulfide isomerase.[19] The additional structural elements surrounding the DSBH active site are commonly involved in 'prime substrate' binding,[8] and there is evidence that elements involved in substrate binding are more flexible than those that are not.[8,20,21] In some cases, *e.g.* γ-butyrobetaine hydroxylase (BBOX) and FIH, the oligomerisation elements are also involved in forming the active site and are likely important for catalysis.[17,18]

In some cases 2OG oxygenases are part of multidomain proteins. This appears to be the case particularly for the JmjC subfamily of histone demethylases which are chromatin modifying 2OG oxygenases that are often linked to one or more histone binding domain modules, which can have important functional roles;[22] in the case of PHF8, an N-terminal plant homeobox domain (PHD domain) module has been shown to be important in catalysis and in targeting the catalytic domain to a particular histone state.[23,24] 2OG oxygenases may also contain other metal binding domains (*e.g.* the zinc-binding BBOX N-terminal domain, plant homeo domain (PHD) fingers and CxxC zinc finger domains) and in at least one case (the JMJD2 subfamily of histone demethylases) a structural Zn(II) binding motif within the 2OG oxygenase domain that is involved in substrate binding.[25–27] Knowledge of how 2OG oxygenases fold and, where applicable, oligomerise, may be a way to obtaining selective inhibitors. One 'proof of principle' study on small molecules that eject zinc from the JMJD2 subfamily suggests that such approaches may be viable.[27] However, the vast majority of 2OG oxygenase inhibitors target the active site iron and its immediate vicinity (see below).

Extensive crystallographic and spectroscopic analyses of 2OG oxygenases provide detailed insights into the iron and cosubstrate binding sites. The active sites contain a very

Table 1 List of assigned or proposed human 2OG oxygenases with substrates. Primary citations are given for recent assignments; see review[7] for citations to other assignments

Gene name	GeneID	Chemical function if assigned
ASPH (aspartyl/asparaginyl β-hydroxylase)	444	Asn/Asp 3*R*-hydroxylase, EGF-like domains
ASPHD2 (hypothetical protein LOC57168)	57168	
ASPHD1 (hypothetical protein LOC253982)	253982	
C17orf101 (PKHD domain-containing transmembrane protein C17orf101)	79701	
LEPRE1, LEPRE1-like, LEPRE2 (leucine proline-enriched proteoglycan (leprecan))	64175 55214 10536	Prolyl-3*S*-hydroxylase
P4H TM (hypoxia-inducible factor prolyl 4-hydroxylase isoform a, transmembrane (endoplasmic reticulum))	54681	Prolyl hydroxylation
P4HA1, P4HA2, P4HA3 (procollagen-proline, 2-oxoglutarate 4-dioxygenase)	5033 8974 283208	Prolyl-4*R*-hydroxylase (collagen)
PLOD1, PLOD3, PLOD2 (procollagen-lysine, 2-oxoglutarate 5-dioxygenase)	8985 5351 5352	Lysyl-5*R*-hydroxylase (collagen)
JMJD4 (Jumonji domain containing 4)	65094	
JMJD6/PTDSR (Jumonji domain containing 6/phosphatidylserine receptor)	23210	Lysyl-5-hydroxylase
JMJD5 (Jumonji domain containing 5/hypothetical protein FLJ13798)	79831	Histone H3K36me2 demethylase[277]
JMJD8 (hypothetical LOC339123)	339123	
TYW5/C2orf60 (chromosome 2 open reading frame 60)	129450	Wybutosine hydroxylase (modified RNA)[79]
FIH (factor inhibiting hypoxia-inducible factor)	55662	Asparaginyl hydroxylase (HIF CTAD and ankyrin repeat domain containing proteins)
PASS1/HSPBAP1	79663	
JMJD7/PLA2gIVB (phospholipase A2, group IVB)	8681	
NO66	79697	Proposed histone demethylase[278]
MINA53B (MYC induced nuclear antigen)	84864	
JMJD3/KDM6B (Jumonji domain containing 3/histone lysine demethylase 6B)	23135	Histone H3K27me3 demethylase
UTX/KDM6A (ubiquitously transcribed tetratricopeptide repeat, X chromosome/histone lysine demethylase 6B)	7403	Histone H3K27me3 demethylase
UTY (ubiquitously transcribed tetratricopeptide repeat protein, chromosome Y)	7404	
JARID1B/PLU-1/KDM5B (Jumonji, AT rich interactive domain 1B (RBP2-like))	10765	Histone H3K4me3 demethylase
JARID1A/RBBP2/KDM5A (retinoblastoma binding protein 2)	5927	Histone H3K4me3 demethylase
JARID1C/SMCX/KDM5C (Smcx homolog, X chromosome)	8242	Histone H3K4me3 demethylase
JARID1D/SMCY/KDM5D (Smcy homolog, Y-linked)	8284	Histone H3K4me3 demethylase
JMJD2A/JHDM3A/KDM4A (Jumonji domain containing 2A/histone lysine demethylase 4A)	9682	Histone H3K9me3/K36me3 demethylase
JMJD2C/GASC1/KDM4C (Jumonji domain containing 2C/gene amplified in squamous cell carcinoma 1/histone lysine demethylase 4C)	23081	Histone H3K9me3/K36me3 demethylase
JMJD2B/KDM4C (Jumonji domain containing 2B/histone lysine demethylase 4B)	23030	Histone H3K9me3/K36me3 demethylase
JMJD2D/KDM4D (Jumonji domain containing 2D/histone lysine demethylase 4D)	55693	Histone H3K9me3 demethylase
JMJD2E/KDM4E (Jumonji domain containing 2E/histone lysine demethylase 4E)		Histone H3K9me3 demethylase
FBXL10/JHDM1B/KDM2B (F-box and leucine-rich repeat protein 10/histone lysine demethylase 2B)	84678	Histone H3K36me2 demethylase
FBXL11/JHDM1A/KDM2A (F-box and leucine-rich repeat protein 11/histone lysine demethylase 2A)	22992	Histone H3K36me2 demethylase
KIAA1718/JHDM1D/KIAA1718	80853	Histone H3K9me2/K27me2 demethylase
PHF8/KIAA1111 (PHD finger protein 8)	23133	Histone H3K9me2 demethylase
PHF2/JHDM1E/GRC5 (PHD finger protein 2)	5253	
HR (hairless)	55806	
JMJD1A/KDM3A/TSGA (Jumonji domain containing 1A/histone lysine demethylase 3A)	55818	Histone H3K9me2 demethylase
JMJD1B/KDM3B/5qNCA (Jumonji domain containing 1B/histone lysine demethylase 3B)	51780	Histone H3K9me2 demethylase
JMJD1C/TRIP8/KIAA1380 (Jumonji domain containing 1C/histone lysine demethylase 3C)	23081	Histone H3K9me2 demethylase
JARID2/JMJ (JARID2/original Jumonji protein)	3720	Missing iron binding residue
PHD1 (HIF prolyl-4-hydroxylase, N-terminal domain disordered)	112398	Prolyl-4*R*-hydroxylase
PHD2 (HIF prolyl-4-hydroxylase, N-terminal MYND)	54583	Prolyl-4*R*-hydroxylase
PHD3 (HIF prolyl-4-hydroxylase, No N-terminal domain)	112399	Prolyl-4*R*-hydroxylase
ABH1 (alkylated DNA repair protein alkB homolog ABH1)	8846	3-meC demethylase (DNA/RNA)
ABH2 (alkylated DNA repair protein alkB homolog ABH2)	121642	1-meA/3-meC demethylase (DNA/RNA)

Table 1 (*continued*)

Gene name	GeneID	Chemical function if assigned
ABH3 (alkylated DNA repair protein alkB homolog ABH3)	221120	1-meA/3-meC demethylase (DNA/RNA)
ABH4 (alkylated DNA repair protein alkB homolog ABH4)	54784	
ABH5 (alkylated DNA repair protein alkB homolog ABH5)	54890	
ABH6 (alkylated DNA repair protein alkB homolog ABH6)	84964	
ABH7 (alkylated DNA repair protein alkB homolog ABH7)	84266	
ABH8 (alkylated DNA repair protein alkB homolog ABH8)	91801	5-Methoxycarbonyl-methyluridine (*S*)-hydroxylase (modified RNA)[77]
FTO (fat mass and obesity associated)	79068	3-meT demethylase (DNA/RNA)
TET1, TET2, TET3 (ten-eleven translocation 1 gene protein)	80312	5-meC hydroxylase (DNA)
	54790	
	200424	
PAHX (phytanoyl-CoA hydroxylase precursor)	5264	Phytanoyl-CoA 2-*threo*-hydroxylase
PHYHD1	254295	
OGFOD1 (2OG, Fe dependent oxygenase domain 1)	55239	
OGFOD2 (2OG, Fe dependent oxygenase domain 2)	79676	
GBBH/BBOX (γ-butyrobetaine hydroxylase)	8424	γ-Butyrobetaine 3*S*-hydroxylase
TMLH (trimethyllysine hydroxylase)	55217	Trimethyllysine 5*R*-hydroxylase

highly, but likely not universally, conserved HXD/E...X_n...H iron binding motif[13,14,28] comprising one aspartyl/glutamyl and two histidyl residues (Fig. 1). There is, however, some evidence that the HXD/E...X_n...H motif may not be fully conserved, with a possibility that only two histidyl iron-binding ligands are required for activity;[29] indeed this is the case for the related 2OG dependent halogenases.[30] This triad of residues forms one half of a metal binding site, with an octahedral (or near octahedral) metal binding geometry. Evidence to support this metal cofactor coordination mode comes from crystal structures for multiple 2OG oxygenases, the first of which was for deacetoxycephalosporin C synthase, DAOCS.[14] It should be noted that the iron is bound less tightly than in the haem oxygenases, and can normally be removed by treatment with appropriate metal chelators, such as EDTA or desferrioxamine or exchanged with excess alternative divalent metals (see below). However, there is evidence that the strength of iron binding varies considerably,[31] and in some cases it may have functional relevance. In the resting state the octahedral metal coordination is completed by two to three water molecules, as observed by crystallographic analyses.

Several enzymes, including the oxidases isopenicillin N-synthase (IPNS)[13] and the plant 'ethylene forming enzyme' aminocyclopropane carboxylic acid oxidase (ACCO)[32] have structures that are closely related to the 2OG oxygenases, but do not utilise 2OG as a cosubstrate. In their catalytic cycles (IPNS, and likely ACCO), the prime substrates bind directly to the active site iron.[21,33,34] This has not been observed for 2OG oxygenase substrates to date, though most reported inhibitors complex to the active site iron.

Although there may be exceptions (*e.g.* deacetoxycephalosporin synthase, DAOCS,[14] and related enzymes that do not use 2OG as a cosubstrate, *e.g.* IPNS),[35,36] a consensus outline mechanism has emerged for 2OG oxygenases. Following binding of ferrous iron, 2OG coordinates the metal in a bidentate manner *via* its 1-carboxylate and 2-oxo groups, likely displacing two ligating water molecules (Fig. 2a). The observed coordination position of the 2-oxo group is near-invariant in the available crystal structures, and is always positioned *trans* to the metal coordinating carboxylate of D/E. In contrast, the position of the 2OG 1-carboxylate varies between being adjacent to the prime substrate binding site (*trans* to the N-terminal histidyl of the HXD/E...X_n...H motif) or being in the available coordination position more on the interior of the protein (*i.e.* *trans* to the C-terminal histidyl of the HXD/E...X_n...H motif). There is evidence that 2OG can 'flip' between these two coordination states/modes.[37] The possibility of different coordination modes needs to be considered in the design of inhibitors that chelate in the active site. Care should also be taken in assuming that coordination modes observed with iron surrogates (*e.g.* nickel, manganese, which are often used for crystallography) apply to iron itself.

In all reported structures of the enzyme·2OG complex, the 2OG C-5 carboxylate group is positioned to form electrostatic interactions with a basic residue (normally a lysine or arginine residue) derived from either DSBH β-strand IV or VII, and is often apparently anchored by at least one additional hydrogen bond to an alcoholic (serine or threonine) or phenolic (tyrosine) residue.[35] From the inhibition perspective it should be noted that although the general mode of 2OG binding appears conserved, there is considerable variation in the 2OG binding sites which can be exploited for selective inhibition.[38,39] The available evidence is that many of the 2OG oxygenase inhibitors developed to date bind in a manner closely related to that of 2OG itself, *e.g.* are bidentate iron chelators. Many, but not all, are functionalised such that they can also exploit the 2OG C-5 carboxylate binding site (see below).

Following 2OG binding, the prime substrate can then bind to the active site, and in doing so weakens binding of the remaining water to the metal, so 'triggering' oxygen binding and reaction.[40] These proposals are supported both by crystallographic and spectroscopic studies.[5,15,40] Oxygen then binds to the Fe(II) and reacts with the iron-bound 2OG in an oxidative decarboxylation process to generate a ferryl intermediate, CO_2 and succinate (Fig. 2a). The ferryl species has been characterised spectroscopically[41–45] and it likely effects hydroxylation *via* a radical rebound process, as proposed for the haem oxygenases.[46] More complex oxidative reactions, *e.g.* rearrangements, are likely to involve further intermediates.

Table 2 Reported crystal structures for 2OG oxygenase inhibitor co-structures. Where substrate is also present this is indicated. Abbreviations: *N*-oxalylglycine (NOG); C-terminal oxygen-dependent degradation domain (CODD) of human HIF-1α isoform; histone H3 lysine in residue *x*, methylation state (*n*) (H3K(*x*)me(*n*)); thymidine-1-*N*-methyladenosine-thymidine (TmAT); 3-methylthymidine (3meT)

Abbreviation	Full name	Species	PDB ID	Reference	Active site metal modelled as	Ligand(s)	Resolution/Å
DAOCS	Deacetoxycephalosporin C synthase	*Streptomyces clavuligerus*	1DCS	279	—	—	1.3
			1E5H	280	Fe^{II}	CO_2, succinate	1.96
			1UO9	281	Fe^{II}	Succinate	1.5
ANS	Anthocyanidin synthase	*Arabidopsis thaliana*	1GP6	282	Fe^{II}	Succinate, quercetin, *trans*-dihydroquercetin, 2-(*N*-morpholino)-ethanesulfonic acid	1.75
FIH	Factor inhibiting hypoxia-inducible factor	*Homo sapiens*	1H2K	283	Fe^{II}	NOG, HIF peptide	2.15
			1H2M	283	Zn^{II}	NOG, HIF peptide	2.5
			1YCI	284	Fe^{II}	*N*-Oxalyl-D-phenylalanine	2.7
			2CGO	158	Fe^{II}	Fumarate	2.4
			2CGN	158	Fe^{II}	Succinate	2.5
			2W0X	102	Fe^{II}	2,4-Pyridinedicarboxylate	2.12
			2WA4	102	Fe^{II}	*N*,3-Dihydroxybenzamide	2.5
			2WA3	102	Fe^{II}	3-Hydroxyphenyl(oxo)acetic acid	2.5
			3OD4	NA	Zn^{II}	8-Hydroxyquinoline	2.2
ATSK	Alkylsulfatase	*Pseudomonas putida*	1VZ4	285	Fe^{II}	Succinate	2.5
			1VZ5	285	—	Succinate	2.15
AlkB	DNA/RNA repair enzyme AlkB	*Escherichia coli*	2FDG	286	Fe^{II}	Succinate, TmAT	2.2
			2FDJ	286	Fe^{II}	Succinate	2.1
PHD2	Prolyl hydroxylase domain isoform 2	*Homo sapiens*	2G1M	241	Fe^{II}	2-[(4-Hydroxy-8-iodoisoquinoline-3-carbonyl)amino]acetic acid	1.7
			2G19	241	Fe^{II}	2-[(4-Hydroxy-8-iodoisoquinoline-3-carbonyl)amino]acetic acid	2.15
			3HQU	93	Fe^{II}	2-[(1-Chloro-4-hydroxyisoquinoline-3-carbonyl)amino]acetic acid, CODD	2.3
			3HQR	93	Mn^{II}	NOG, CODD	2.0
			2HBT	NA	Fe^{II}	2-[(1-Chloro-4-hydroxyisoquinoline-3-carbonyl)amino]acetic acid	1.6
			2HBU	NA	Fe^{II}	2-[(1-Chloro-4-hydroxyisoquinoline-3-carbonyl)amino]acetic acid	1.85
P4H	Prolyl 4-hydroxylase	*Chlamydomonas reinhardtii*	2JIG	107	Zn^{II}	2,4-Pyridinedicarboxylate	1.85
JMJD2A	JmjC domain containing protein 2A	*Homo sapiens*	2OX0	26	Ni^{II}	NOG, H3K9me2	1.95
			2OT7	26	Ni^{II}	NOG, H3K9me1	2.13
			2OS2	26	Ni^{II}	NOG, H3K36me3	2.3
			2OQ7	26	Ni^{II}	NOG	2.15
			2OQ6	26	Ni^{II}	NOG, H3K9me3	2
			2Q8E	67	Ni^{II}	NOG, H3K36me3	2.05
			2Q8D	67	Ni^{II}	Succinate, H3K36me2	2.3
			2PXJ	146	Fe^{II}	NOG, H3K36me1	2
			2P5B	146	Fe^{II}	NOG, H3K36me3	2.06
			2VD7	68	Ni^{II}	2,4-Pyridinedicarboxylate	2.25
			2WWJ	95	Ni^{II}	*O*-Benzyl-*N*-(carboxycarbonyl)-D-tyrosine	2.6
			3NJY	70	Ni^{II}	5-carboxy-8-hydroxyquinoline	2.6
JMJD2E	LOC390245	*Homo sapiens*	2W2I	NA	Ni^{II}	Glycerol, 2,4-pyridinedicarboxylate	2.1
PHF8	PHD finger protein 8	*Homo sapiens*	3KV4	23	Fe^{II}	NOG, H3K4me3K9me2	2.19
KIAA1718	JmjC domain-containing histone demethylation protein 1D	*Homo sapiens*	3KV5	23	Fe^{II}	NOG	2.39
JMJD3	JmjC domain containing protein 3	*Homo sapiens*	2XXZ	NA	Ni^{II}	5-carboxy-8-hydroxyquinoline	1.8
BBOX1	γ-Butyrobetaine hydroxylase	*Homo sapiens*	3MS5	17	Ni^{II}	3-(2,2,2-Trimethylhydrazine)-propionate	1.82
FTO	Fat mass and obesity associated protein	*Homo sapiens*	3LFM	287	Fe^{II}	NOG, 3meT	2.5

The point at which CO_2 leaves the active site is unclear. It is also important to note that there is evidence that the rate limiting step varies,[47] and that in some cases product release may be limiting.[48] Indeed, in some cases product, substrate and/or cosubstrate inhibition has been observed. However, there are relatively few examples of prime substrate-based

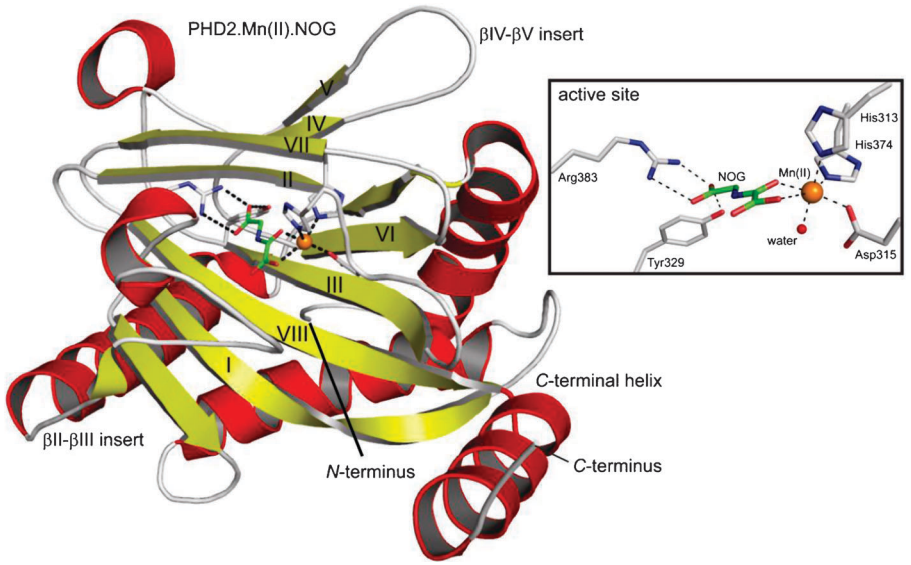

Fig. 1 General structural features of 2OG oxygenases. The view is derived from a crystal structure of the catalytic domain of the hypoxia inducible factor prolyl hydroxylase PHD2 (EGLN1) PDB ID 3HQR. The eight strands of the conserved double-stranded β-helix core fold are labelled I to VIII. Mn(II) and *N*-oxalylglycine (NOG) substitute for Fe(II) and 2OG respectively. The inset is a close-up of the active site showing the metal and 2OG binding residues.

inhibitors of the 2OG oxygenases. One example is the clinically used BBOX inhibitor, THP, which is a substrate-competitive analogue which is itself oxidised by BBOX (see below).[17]

Although our review focuses on inhibitors of the 2OG oxygenases, it should be noted that methods for increasing their 2OG oxygenase activity are of therapeutic interest in some cases (*e.g.* the hypoxia inducible factor (HIF) hydroxylases in tumours—see below). The activity of most, but not all, 2OG oxygenases is stimulated by reducing agents, including ascorbate (Vitamin C). Lack of ascorbate in the human diet is proposed to cause the disease scurvy, famously studied by James Lind in the 18th century,[49] who recognised it could be treated by the addition of fresh fruits and vegetables to the diet. Because the activity of the collagen prolyl hydroxylases is stimulated by ascorbate, and because humans, unlike many other animals, do not biosynthesise ascorbate, it is proposed that ascorbate deficiencies result in impaired collagen formation.[3] However, the precise mechanism by which ascorbate enhances 2OG oxygenase activity is unknown and may involve more than one process, *e.g.* reduction of catalytically inactive iron oxidation states. Further, recent work has shown that the role of ascorbate in 2OG oxygenases catalysis can be replaced by glutathione (and likely by other reducing agents).[50] Thus, further investigation is required in this field.

2OG decarboxylation without (or with partial) concomitant prime substrate oxidation has been observed for many 2OG oxygenases.[5,51] This 'uncoupled' turnover can be increased by substrate analogues and active site substitutions. In some cases, (AlkB, TauD) the substrate-uncoupled turnover of 2OG results in auto-oxidation of aromatic residues in the active site in a process that may also be mediated by the reactive Fe(IV)=O species.[52,53] It is proposed (particularly for the collagen prolyl-4-hydroxylases)[54] that one role for ascorbate is to reduce Fe(III) or Fe(IV) produced as a result of these uncoupled reaction cycles, to regenerate the catalytically active Fe(II) species.

3. Assays for 2OG oxygenases

The conserved role of 2OG, oxygen and succinate/CO_2 as cosubstrates/products for 2OG oxygenases has enabled the development of assays that are, in principle, generic (Table 3). The most widely used of these are based on the production of radiolabelled CO_2 or succinate.[55–59] Other assays have included (i) 2OG consumption by derivatisation,[60,61] (ii) spectroscopic[41,43,47,48] or electrochemical detection[62,63] of oxygen consumption, and (iii) LC(MS) detection of succinate (Table 3).[58] The ^{14}C-labelled CO_2 detection assay has been extensively used in academic laboratories, and detection of ^{14}C-labelled succinate has been developed and applied to inhibitor screening.[61] Enzyme-coupled assays for succinate detection have also been developed, employing succinate dehydrogenase[64] or succinyl-CoA synthetase together with pyruvate kinase and lactate dehydrogenase. The usefulness of these enzyme-coupled approaches depends on the compatibility of the kinetic properties of the coupled enzymes with the 2OG oxygenase under investigation.[65]

Assays specific to particular 2OG oxygenases rely on detection of hydroxylated products or, in the case of the demethylases, detection of the coproduct formaldehyde.[66] In the latter case, assays that use formaldehyde dehydrogenase to couple the oxidation of formaldehyde to the reduction of NAD^+ permit spectroscopic detection of NADH (produced by the coupled enzyme) to monitor demethylation.[23,67–69] This assay method has been successfully applied to high-throughput inhibitor screening of a histone demethylase.[70,71] Assays for hydroxylated (or demethylated) products have employed MS,[50,68] NMR[47] or antibody-based methods including using

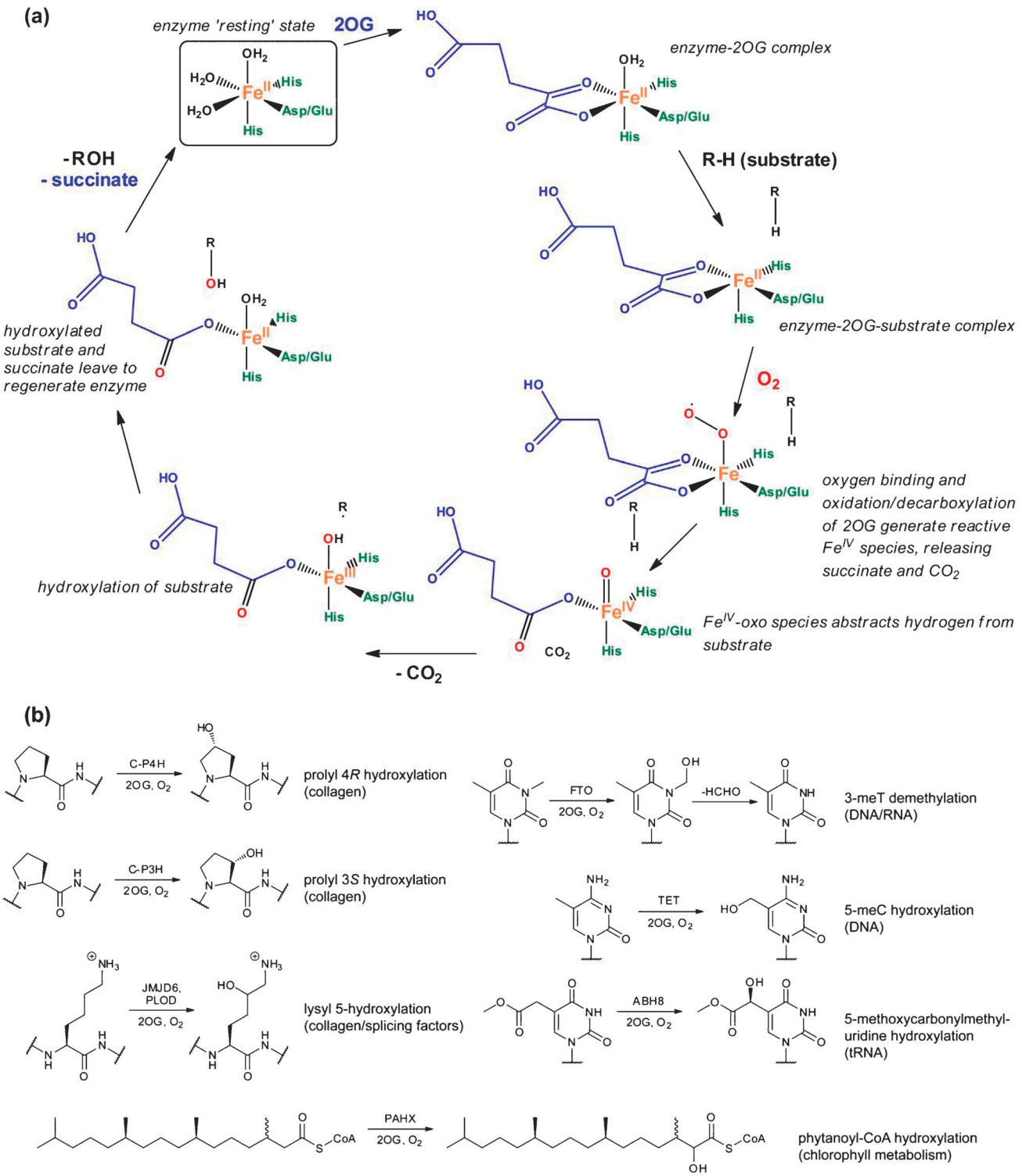

Fig. 2 Outline consensus mechanism for 2OG oxygenases and selected reactions catalysed by 2OG oxygenases. (a) There is evidence for all the intermediates shown, including the ferryl species, except for the O_2 complex and the Fe(III)–OH intermediate shown after reaction of the ferryl species. (b) Selected reactions catalysed by human 2OG oxygenases, showing the range of substrate types accepted by these enzymes. Other human 2OG oxygenase reactions are in Fig. 10, 12 and 15.

luminescence/fluorescence-based methodology suitable for high-throughput screening (*e.g.* time-resolved fluorescence resonance energy transfer-based assay (TR-FRET),[72] and Amplified Luminescence Proximity Homogenous Assay (ALPHAscreen)).[73]

As for many pharmaceutical targets, there is a current need for reliable and quantitative cell-based assays for 2OG oxygenases. Early studies demonstrated the use of cell-based assays for studying collagen prolyl hydroxylation.[74] More recently,

Table 3 Assays employed for 2OG oxygenases. Note that although many of the assays below are applicable in principle to all 2OG oxygenases, in practice this is not the case due to limitations imposed by detection limits and/or kinetic parameters. Exemplary references are given for each method

Assay method	Detection of	Analytical method	Applicable to	Comments
Derivatisation of unreacted 2OG with *o*-phenylenediamine[60]	2OG	Spectrophotometric fluorescent measurement of *o*-phenylenediamine-2OG adduct	All 2OG oxygenases	Ascorbic acid interferes with assay
Glutamate dehydrogenase conversion of 2OG to glutamate[288]	2OG	Spectrophotometric measurement of NAD^+	All 2OG oxygenases	
Decarboxylation of 1-^{14}C-2OG[56,57]	Carbon dioxide	Radioisotope measurement of $^{14}CO_2$	All 2OG oxygenases	
Measurement of soluble 4-^{14}C-succinate after precipitation of unreacted 5-^{14}C-2OG[58,59,61]	Succinate	Radioisotope measurement of 4-^{14}C-succinate	All 2OG oxygenases	
LCMS detection of succinate[47]	Succinate	LCMS	All 2OG oxygenases	
Succinate dehydrogenase-coupled oxidation of succinate[64]	Succinate	Spectrophotometric measurement of reduced cofactors	All 2OG oxygenases	Succinate dehydrogenase reportedly unstable[65]
Succinyl-coenzyme A synthetase, pyruvate kinase, and lactate dehydrogenase coupled detection of succinate[65]	Succinate	Spectrophotometric absorbance measurement of NAD^+	All 2OG oxygenases	Use of coupled assays can complicate kinetic inhibition studies.
^{3}H-release from radiolabelled substrates during hydroxylation[42,160]	^{3}H	Radioisotope measurement of ^{3}H	All 2OG oxygenases	Relies on synthesis of suitably radiolabelled substrates—often difficult/ expensive
(LC)-MS of product peptides/ proteins/small molecules[50,68]	Hydroxylated/ demethylated product peptides/proteins	Mass spectrometry, possibly coupled with liquid chromatography	All 2OG oxygenases	Has been used for inhibition assays[68]
NMR[289]	2OG/succinate substrates with products	^{1}H NMR	All 2OG oxygenases	Can be used to measure coupling efficiency but labour intensive
Western blotting of product peptides/proteins[66,290]	Hydroxylated/ demethylated product peptides/proteins		2OG oxygenases with peptide/protein substrates	Relies on availability of suitable antibodies
Derivatisation of radiolabelled formaldehyde as demethylation product[66]	Formaldehyde	Radioisotope measurement of Nash Reaction-derivatised radiolabelled formaldehyde	Histone and DNA demethylases	Relies on synthesis or enzymatic radiolabelling of substrates
Formaldehyde dehydrogenase (FDH)-coupled oxidation of formaldehyde[23,68,69,157]	Formaldehyde	Spectrophotometric fluorescent measurement of NADH/APADH	Histone and DNA demethylases	Has been used for inhibition assays (including high throughput)[71,291]
Capillary electrophoresis separation of methylated/ demethylated oligonucleotides[292]	Demethylated oligonucleotides	Capillary electrophoresis/ spectrophotometric detection of fluorescently labelled oligonucleotides	DNA demethylases	Has been used for inhibition assays, likely applicable to protein demethylases
Homogeneous time resolved fluorescence (HTRF) detection of hydroxylated peptide/protein product[38,72]	Eu^{3+}-cryptate labelled von Hippel–Lindau-Elongin B–Elongin C complex binding to hydroxylated peptide substrate	Time-resolved fluorescence of Eu^{3+}-cryptate	HIF prolyl hydroxylases	Has been used successfully for inhibition assays
Amplified Luminescence Proximity Homogeneous Assay (ALPHA) Screen[73]	Detection of methylated peptide substrate/product	Luminescence using ALPHA beads and antibodies against methylated substrate/ product	Histone demethylases (adaptable to oxygenase with peptide/protein substrate	Relies on availability of suitable antibodies. Has been used successfully for inhibition assays.

Table 4 Reported IC_{50} or K_i values (in parentheses) for *N*-oxalyl amino acids and related compounds against human and selected bacterial 2OG oxygenases. See Table 1 for definition of the 2OG oxygenases, and Fig. 3 for inhibitor structures

	C-P4H	PHD1	PHD2	PHD3	FIH	AlkB	JMJD2E	PAHX
N-Oxalylglycine	(0.5 μM)[87] (1.9 μM)[86]	(50 μM)[186]	(8 μM)[186] 19 μM[38]	(10 μM)[186]	(2 μM)[89]	0.7 mM[92]	75 μM[87]	Inhibits
N-Oxalyl-D-Ala	621 μM[87]		Does not inhibit[94]		Inhibits[94]	2.39 mM[92]	29 μM[95]	
N-Oxalyl-L-Ala	38 μM[87]		Inhibits[94]		Inhibits[94]	3.33 mM[92]	Does not inhibit[95]	
N-Oxalylcysteine	Inhibits[86] (racemic?)						D-Enantiomer 73 μM, L-does not inhibit[95]	
N-Oxalyl-β-alanine	Inhibits[86]							
2-Thiono-*N*-oxalylglycine						0.81 mM[92]		
2-Mercaptopentanedioic acid						0.12 mM[92]		
N-Oxalyl-D-phenylalanine	<18% at 200 μM		Does not inhibit[94]		(83 μM)[94]		320 μM[68]	

Table 5 Table of reported IC_{50} or K_i values (in parentheses) for 2,4- and 2,5-pyridinedicarboxylate against human 2OG oxygenases. See Table 1 for definition of the 2OG oxygenases, and Fig. 7 for inhibitor structures

	C-P4H	C-P3H	PHD1	PHD2	PHD3	FIH	JMJD2E
2,4-PDCA	(2 μM)[42]	(3 μM)[160]	(40 μM)[186]	(7 μM)[186]	(8 μM)[186]	(30 μM)[89]	1.4 μM[68]
2,5-PDCA	(0.8 μM)[42]	(15 μM)[160]	No inhibition at 300 μM[186]	No inhibition at 300 μM[186]	No inhibition at 300 μM[186]	(50 μM)[89]	180 μM[68]

Table 6 Table of available inhibition data for flavonoid/catechol inhibitors of 2OG oxygenases. IC_{50} or K_i values are shown, with K_i values in parentheses. See Table 1 for definition of the 2OG oxygenases, and Fig. 6 for inhibitor structures

	C-P4H	PLOD1	PHD1	PHD2	PHD3	FIH	AlkB	JMJD2E
Quercetin				Inhibits at 50 μM[120,293]		0.6 mM[92]	80 μM[92]	
Baicalein				7 μM[119]		860 nM[119]		(4.33 μM)[71]
Epinephrine		(0.2–0.5 mM)[123]						
3,4-Dihydroxybenzoate	(5 μM)[124]		No inhibition at 300 μM[186]	Inhibits at 100 μM[294]/ no inhibition at 300 μM[186]	No inhibition at 300 μM[186]	(10 μM)[89]		
Gallate				~0.3 mM[125]				
2,7,8-Trihydroxy-anthraquinone	(40 μM)[295]							

assays have been reported for HIF prolyl hydroxylation and histone demethylation using western blotting, reporter gene-based luminescence,[75] antibody-based immunofluorescence[70] and mass spectrometric approaches.[76]

It should be stated that because of the range of assays and conditions that have been used for various 2OG oxygenases, care should be taken in comparing different sets of inhibition data (including the data given in Tables 4–6). Thus, while robust assays exist for individual 2OG oxygenases, the lack of generic, high-throughput methods for measuring inhibitor potencies *in vitro* is a limiting factor in understanding selectivity (as is the case for many enzyme families).

4. Biological roles of 2OG oxygenases

Following the pioneering work on the collagen prolyl hydroxylases, substantial progress has been made in defining other roles for human 2OG oxygenases (Table 1, Fig. 2b). Prolyl (PHDs) and asparaginyl (FIH) hydroxylases play important roles in hypoxic sensing (see below). 2OG oxygenases have also been shown to catalyse hydroxylation of Asp/Asn residues in epidermal growth factor-like domains (ASPH), ankyrin repeat domains (the asparaginyl hydroxylase FIH) and splicing regulatory proteins (the lysyl hydroxylase JMJD6). They also modify proteins *via* N^{ε}-demethylation of N^{ε}-methylated histone lysine residues, in a reaction proceeding *via* N^{ε}-methyl hydroxylation (see below). 2OG oxygenases play important roles in fatty acid metabolism, both in terms of carnitine biosynthesis (see below) and in the metabolism of phytanic acid, which is derived from the fatty acid sidechains of chlorophyll (Fig. 2b).

Finally, 2OG oxygenases have emerged as an important class of nucleic acid modifying enzymes (Fig. 2b), both as demethylases in repair processes (the enzymes ABH2, ABH3, and FTO), and as hydroxylases of modified RNA (ABH8[77,78] and TYW5, or wybutosine hydroxylase)[79] and 5-methylcytosine (TETs).[80] In the case of the nucleic acid demethylase FTO, mutations in the 2OG oxygenase domain have physiological relevance, in that they are associated with increased fat mass and obesity.[81,82]

5. 'Generic' inhibitor templates

In this section we outline some general themes in work on the inhibition of 2OG oxygenases. The vast majority of such compounds are predicted to inhibit by chelating to the active site Fe(II) and hence will compete with 2OG and possibly with oxygen for binding. Most of the potent inhibitors also likely

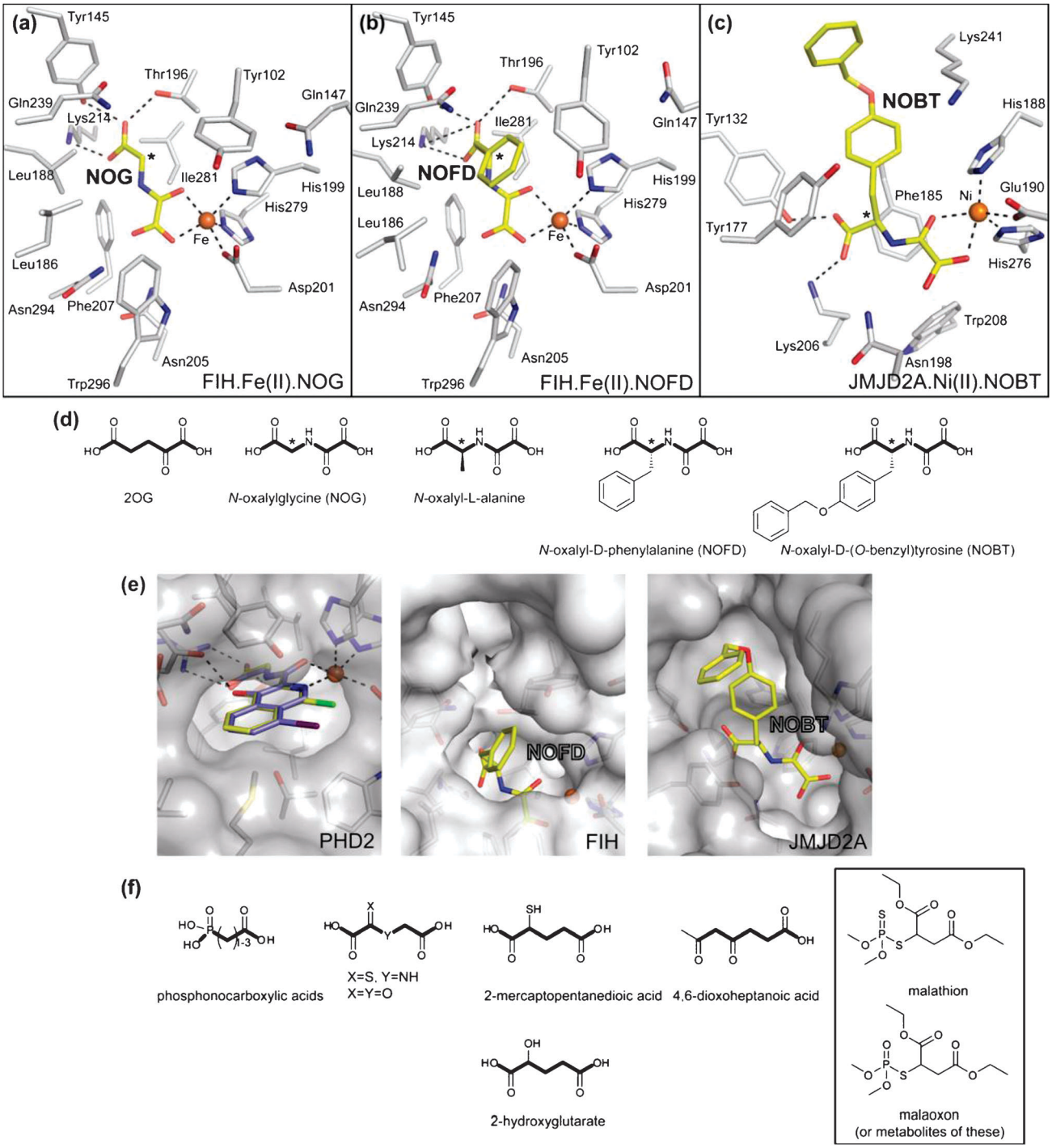

Fig. 3 *N*-Oxalyl amino acids are 'generic' inhibitors of 2OG oxygenases, binding in a similar mode to the 2OG cosubstrate. Differences in the 2OG and substrate binding pockets can be exploited for selective inhibition. (a) View from a crystal structure of FIH complexed with *N*-oxalylglycine (PDB ID 1H2K). (b) View from a crystal structure of FIH in complex with *N*-oxalyl-D-phenylalanine (PDB ID 1YCI), which inhibits FIH but not PHD2. (c) View from a crystal structure of JMJD2A in complex with NOBT (*N*-oxalyl-D-(*O*-benzyl)tyrosine) which inhibits JMJD2A but not PHD2 (PDB ID 2WWJ). (d) Structures of 2OG and *N*-oxalyl amino acids. (e) Surface views from crystal structures of PHD2 (PDB IDs 2G1M overlayed with 3HQU), FIH (PDB ID 2HBU) and JMJD2A (PDB ID 2WWJ), demonstrating the size and shape of the active site and the scope for variation by designing *N*-oxalyl amino acids with suitable stereochemistry and substituents at the C-α carbon (indicated with * in (a), (b) and (c)). Note that the size of the 2OG binding pocket/active site opening increases on going from PHD2 to FIH to JMJD2A. The PHD2 structure is an overlay of complexes with *N*-[(4-hydroxy-8-iodoisoquinolin-3-yl)carbonyl]glycine and *N*-[(1-chloro-4-hydroxyisoquinolin-3-yl)carbonyl]glycine. (f) Other acyclic analogues of 2OG reported as inhibitors (or precursors of inhibitors).

hinder substrate binding in the active site region. Several generic template 2OG oxygenase inhibitors have been identified that bind in a similar manner to 2OG itself, including *N*-oxalyl amino acids, 2,*x*-pyridinedicarboxylates, and substituted

hydroxamic acids. These compounds act as non-reactive analogues of 2OG, and their functionalisation enables selective inhibition, either by increasing potency towards a particular 2OG oxygenase or by blocking binding to another oxygenase.

N-Oxalylglycine (NOG) and its cell-penetrating derivative dimethyl oxalylglycine (DMOG)[83] have found extensive use as generic inhibitors of 2OG oxygenases (Fig. 3). DMOG has been used in cell culture and in animal models as a non-toxic prodrug form of *N*-oxalylglycine to inhibit the collagen and HIF prolyl hydroxylases, in the latter case acting as a good, but imperfect, mimic of hypoxia.[84,85] NOG was originally identified as an inhibitor of C-P4H, but has subsequently been shown to inhibit a range of enzymes *via* 2OG competition, as shown by kinetic and structural studies (Table 4).[38,68,86–92] Co-crystal structures have been determined for *N*-oxalylglycine in complex with FIH, PHD2, JMJD2A and PHF8.[18,23,26,93] NOG occupies the 2OG binding site, chelating Fe(II) through its amido and C-1 carboxylate oxygens, analogous to chelation by the 2OG ketone and C-1 carboxylate. However, the NOG complex does not react with oxygen, presumably in part because the ketone of 2OG is substituted with a less reactive amide.

Substitution at the C-α position of NOG can lead to increases in the potency and, of particular interest, selectivity of inhibition (Fig. 3). In the case of both the HIF and the collagen prolyl hydroxylases,[83,86,87,94] *N*-oxalyl-L-alanine is a more potent inhibitor than its D-enantiomer, with bulkier substituents being inactive or less potent (Table 4).[87] Notably, the preference of the HIF prolyl hydroxylases (PHDs) for inhibition by the L-alanyl derivatives also extends to cell-penetrating bicyclic aromatic amino acid derivatives.[38] In contrast, as predicted by structural studies, FIH is more potently inhibited by the D-enantiomers,[94] as are the JMJD2 histone demethylases (Table 4).[95] The bacterial enzyme AlkB is inhibited by both enantiomers of *N*-oxalyl amino acids with equal potency.[96]

These studies on *N*-oxalyl amino acid derivatives are important because they demonstrate that differences in the 2OG-binding pockets and adjacent regions can be exploited for selective inhibition. However, a drawback of these compounds is that because they are di-acids, they likely have to be used in prodrug ester form in order to be useful *in vivo*. In the case of the HIF prolyl hydroxylases, related compounds in which the carboxylate or the *N*-oxalyl group is replaced with a heterocycle have been developed and are active in cells and in animals (see below).

Hydroxamic acids are well-established transition metal chelators, especially for Zn(II) enzymes, and have also been shown to inhibit 2OG oxygenases. In addition to iron complexation, some of the reported hydroxamic acid inhibitors bind in the 2OG binding pocket (Fig. 4), whilst others likely

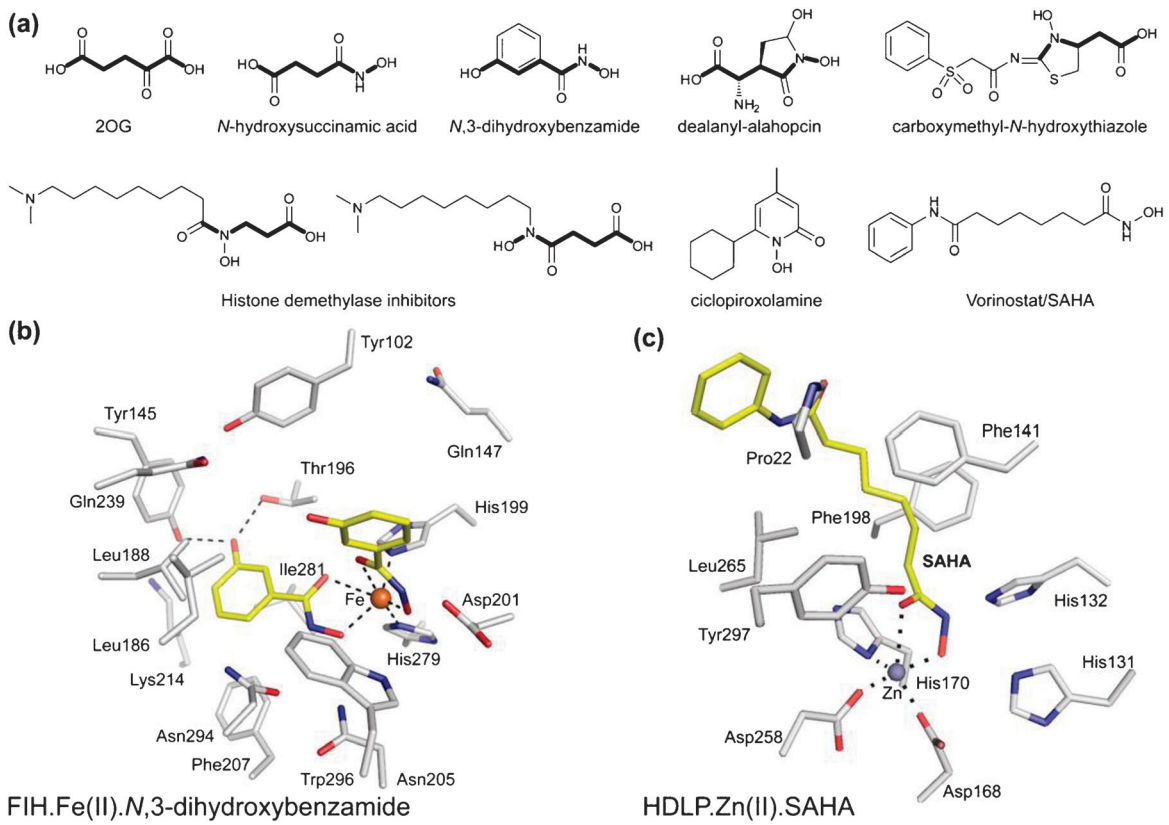

Fig. 4 Hydroxamic acids are inhibitors of 2OG oxygenases. (a) Structures of 2OG and of hydroxamic acids and related compounds reported to inhibit 2OG oxygenases. (b) View from the crystal structure of FIH in complex with 3-hydroxybenzamic acid (PDB ID 2WA4), which occupies the 2OG binding site and chelates the active site metal *via* the hydroxamic acid carbonyl and hydroxyl oxygens. Note that in this structure, another molecule of the hydroxamic acid was observed to bind, displacing the Fe-chelating residue Asp201, which forms part of the conserved HXD···H motif common to most 2OG oxygenases. (c) View from the crystal structure of Histone Deacetylase-Like Protein (HDLP) in complex with SAHA, the first hydroxamic acid HDAC inhibitor to be approved for clinical use (PDB ID 1C3S). As observed in (b), SAHA binds the active site Zn(II) ion through the carbonyl and hydroxyl oxygens of the hydroxamic acid moiety. SAHA has also been observed to inhibit the JMJD2 histone demethylase subfamily.

bind towards the substrate binding pocket. The cyclic hydroxamates ciclopiroxolamine, alahopcin and dealanylalahopcin and analogues have been shown to inhibit C-P4H[97,98] and PHD2 using *in vitro* and in cellular assays.[75,99,100] The structurally related *N*-hydroxythiazoles are some of the most potent PHD2 inhibitors yet identified, with IC_{50} values < 100 nM,[72] while several different hydroxamic acid templates have been shown to inhibit the JMJD2 histone demethylases and FIH.[68,101,102] No selectivity data for these hydroxamic acids have been reported.

Interestingly, in the case of the JMJD2 histone demethylases, hydroxamic acids including the natural product trichostatin A, and the clinically-used Vorinostat (suberoylanilide hydroxamic acid, SAHA) act as inhibitors.[68] Although the degree of inhibition for these compounds against the demethylases is less than for the histone deacetylases (HDACs), these observations suggest that some of the *in vivo* effects of the activity of the hydroxamic acid HDAC inhibitors may be *via* inhibition of the 2OG dependent histone demethylases, or of other 2OG oxygenases. Crystallographic analyses (Fig. 4) with FIH confirm that the hydroxamate moiety chelates the active-site metal (PDB ID 2WA4), as observed for Zn-containing enzymes,[102] including histone deacetylases. It should be noted that as with other Fe-chelators it is important to verify that inhibition by hydroxamic acids occurs *via* active site binding rather than by chelation of iron in solution only.

Various other acyclic 2OG analogues have been reported as inhibitors of 2OG oxygenases (Fig. 3). These include the naturally occurring compounds succinate (a coproduct), fumarate, and 2-hydroxyglutarate (see below), as well as phosphonocarboxylic acids and boronic acids.[83] The phosphonocarboxylic acid result is interesting because the related compounds malathion and malaoxon have been reported to act as prolyl and lysyl hydroxylase inhibitors *in vitro* and in cells.[103–105] Other compounds that are structurally related to 2OG, such as 4,6-dioxoheptanoic acid, have also been reported to inhibit PHD2 *in vitro*,[106] though no selectivity data for other 2OG oxygenases have been reported.

Many cyclic 2OG analogues have been developed as inhibitors (Fig. 5), of which the 2,*x*-pyridinedicarboxylates were a pioneering example. These compounds chelate iron *via* their pyridinyl nitrogen and 2-carboxylate groups. All characterised 2OG oxygenases have a basic residue which 'anchors' the C-5 carboxylic acid of 2OG; likewise the C-4 carboxylate of 2,4-pyridinedicarboxylate (2,4-PDCA) (and in possibly in some cases, 2,5-pyridinedicarboxylate) is anchored in the active site by the same basic residues (this has been demonstrated by structural studies of 2,4-PDCA in complex with C-P4H, FIH and JMJD2A) (Fig. 5).[68,102,107] However, 2,*x*-pyridinedicarboxylates have now been shown to inhibit many other 2OG oxygenases, including the HIF hydroxylases PHD1-3 and FIH, and the histone demethylases (Table 5), as well as the plant gibberellin oxidising enzymes[108] and flavanone 3β-hydroxylase.[109] Given the similarity between 2,4-PDCA and 2OG, it is likely that 2,4-PDCA is a reasonably generic 2OG oxygenase inhibitor, in a similar manner to *N*-oxalylglycine. However, 2,5-PDCA displays some selectivity; it is a relatively potent inhibitor of the collagen prolyl hydroxylases and FIH, but does not inhibit the HIF prolyl hydroxylases (Table 5). Other pyridine dicarboxylates (2,3-, 3,5- and 3,4-pyridinedicarboxylate) inhibit the studied 2OG oxygenases much more weakly,[42,68] while 2,6-PDCA shows an

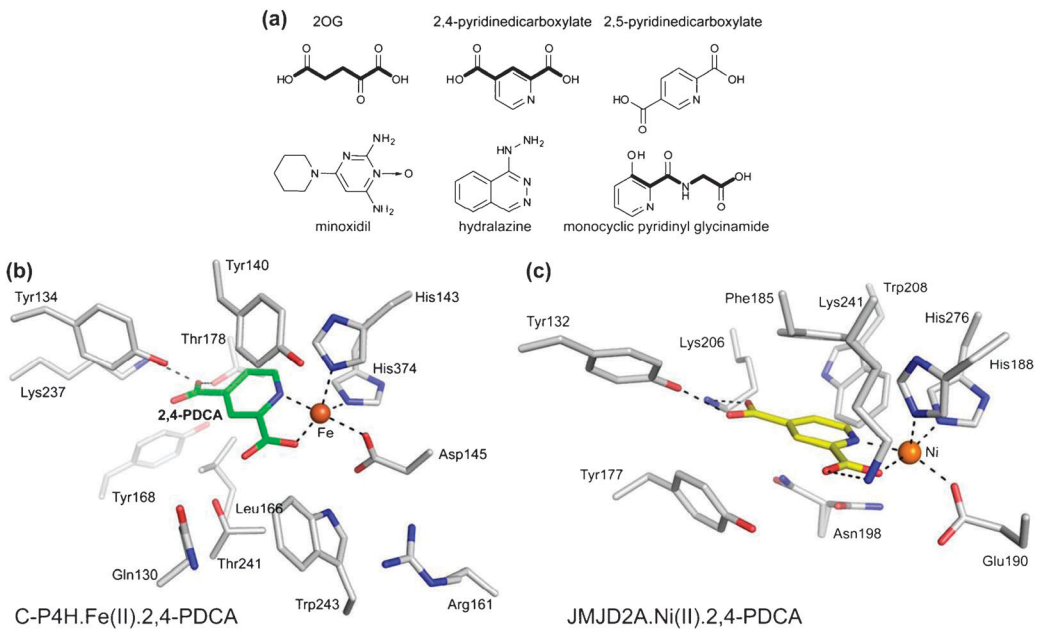

Fig. 5 Pyridine carboxylate inhibitors are simple cyclic analogues of 2OG. (a) Structures of 2OG, 2,4-pyridinedicarboxylate and 2,5-pyridinedicarboxylate, and of the related cyclic inhibitors minoxidil (which inhibits PLOD1), hydralazine (which inhibits C-P4H and the HIF PHDs) and a monocyclic pyridinyl glycinamide (an inhibitor of C-P4H and the HIF PHDs). (b) View from crystal structure of collagen prolyl-4-hydroxylase (C-P4H) from *C. reinhardtii* in complex with 2,4-PDCA (PDB ID 2JIG). Although 2,5-PDCA is reported to be a more potent inhibitor of C-P4H,[42] based on this crystal structure it may bind differently to the manner observed for 2,4-PDCA. (c) View from the crystal structure of the histone demethylase JMJD2A in complex with 2,4-PDCA (PDB ID 2VD7). 2,4-PDCA is positioned to form a salt bridge *via* its C-4-carboxylate with Lys206 (as does 2OG) and chelates the active site metal through the pyridinyl nitrogen and 2-carboxylate, but binds in a different plane to 2OG/NOG (compare PDB ID 2OQ6).

inhibitory effect that is dependent on iron concentration in solution, consistent with its role as a potent iron chelator.[42]

Several other monocyclic iron chelators have been shown to inhibit 2OG oxygenases, including 2-glycinamide derivatives of 3-hydroxypyridine (Fig. 5).[110,111] Other related compounds have also been demonstrated to inhibit some 2OG oxygenases. The pyrimidine-*N*-oxide minoxidil (currently used as a topical treatment for androgenic alopecia)[112] is also an inhibitor of the collagen lysyl hydroxylase PLOD1; for inhibition, the pyrimidine ring can also be replaced by pyridine or *sym*-triazine aromatic heterocycles, providing the 2,5-diamino and *N*-oxide moieties are retained.[113–115] Treatment of human cells with the smooth muscle relaxant hydralazine leads to upregulation of HIF-α PHD target genes in a mechanism proposed to be *via* PHD inhibition.[116] Inhibition of collagen hydroxylation in cells treated with hydralazine has also been reported.[117] Inhibition of the histone and DNA demethylases by hydralazine is also possible (and hydralazine also inhibits the DNA methyltransferase DNMT1),[118] suggesting that hydralazine may affect transcription by multiple mechanisms (though no selectivity data have been reported).

Subsequent to the original studies on acyclic and monocyclic 2OG analogues, various heterocyclic inhibitors of 2OG oxygenases have been developed, in particular for the HIF and collagen prolyl hydroxylases. Past research on these compounds is described in more detail below in the sections on individual enzymes.

6. Inhibition by natural products

2OG oxygenases are involved in the biosynthesis of many natural products, including the metal-chelating flavonoids; hence it could be argued that natural products might be a useful source of inhibitors for 2OG oxygenases. In general, the pharmaceutical industry has not focused on natural products in the last two decades. However, natural products have proven to be a useful source of inhibitors for the histone modifying enzymes, especially histone deacetylases (*e.g.* trichostatin A, which is a generic Zn-dependent HDAC inhibitor), and may also be a useful source for inhibitors of the 2OG oxygenases that modify chromatin. Several natural products have been identified that inhibit the collagen prolyl hydroxylases, including the hydroxamate alahopcin.[98] Derivatives of these compounds have also been shown to inhibit the HIF hydroxylases, likely *via* competition with 2OG.[100]

Various natural product families have been identified from the two publicly available high-throughput screens for 2OG oxygenase inhibitors, both carried out on the JMJD2 histone demethylases (see below for details).[70,71] However, the largest family of natural products to be identified as 2OG oxygenase inhibitors are the flavonoids (Fig. 6, Table 6). Several other reports have also indicated that flavonoids can inhibit 2OG oxygenases and/or induce HIF-α in cells or animal models. Baicalein inhibits PHD2, FIH and JMJD2E *in vitro,* in a manner that was competitive with 2OG for PHD2 and FIH, but noncompetitive for JMJD2E (Table 6).[71,119] Other reports have shown that epicatechin gallate, quercetin, 3′,4′-dihydroxyflavone, 3-hydroxyflavone and galangin are also able to stabilise HIF-α, presumably by inhibition of the prolyl hydroxylases.[119–121] Quercetin also inhibits the DNA demethylase AlkB in a manner that could be reversed by addition of excess iron.[92]

Flavonoid related compounds, such as catechols (including 3,4-dihydrobenzoic acid, epinephrine, gallic acid and catechol itself), have been shown to inhibit 2OG oxygenases (Fig. 6). These compounds may inhibit at least in part by sequestering

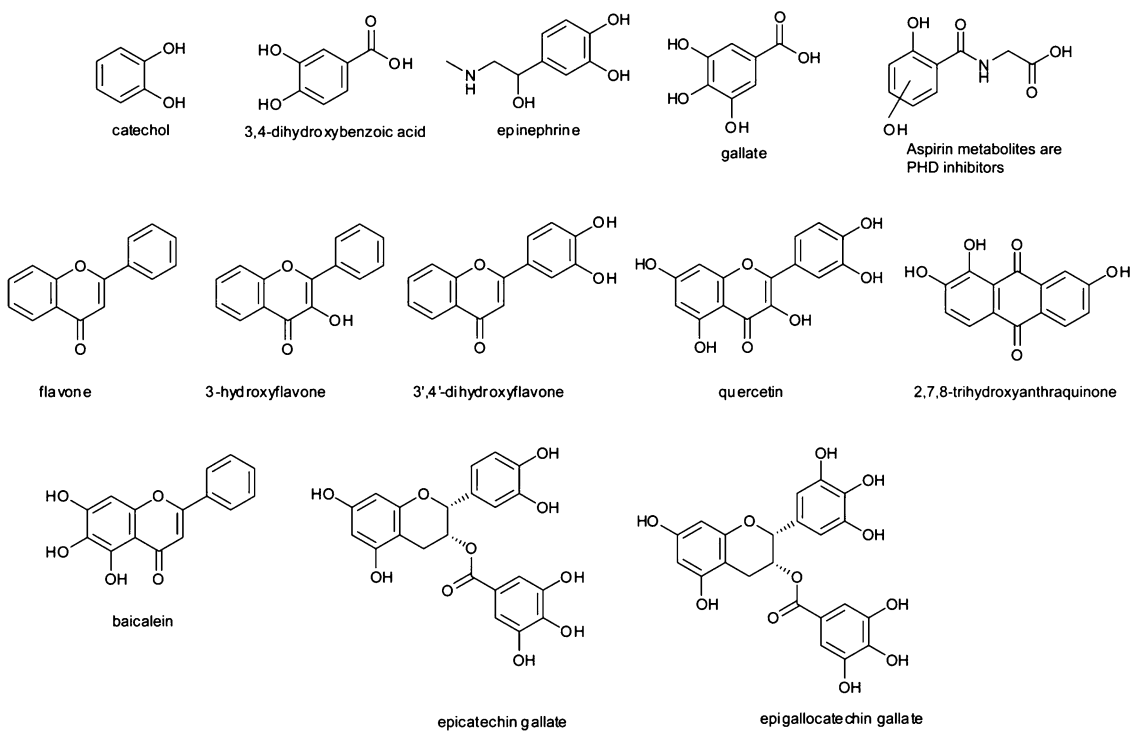

Fig. 6 Flavonoid inhibitors of 2OG oxygenases. Structures of flavonoids and other catechol type compounds observed to inhibit 2OG oxygenases.

iron in solution, or even removing iron from the active site, or they may bind to iron in the active site in a manner competitive with 2OG or substrate. However, flavonoids are also known to act as 'non-specific' inhibitors and may inhibit by affecting protein structure/aggregation.[122] Epinephrine was shown to be noncompetitive in its inhibition of PLOD1 with respect to 2OG and peptide substrate,[123] suggesting a role in chelating iron in solution. In contrast, 3,4-dihydroxybenzoic acid and gallic acid, and several other catechols, are competitive inhibitors of C-P4H with respect to 2OG and ascorbate.[124] Ethyl 3,4-dihydroxybenzoate was effective in reducing collagen production in keloid fibroblasts, by inhibition of C-P4H. Gallic acid, but not its propyl ester, was also demonstrated to inhibit PHD2 hydroxylation of HIF-1α in cell-free extracts[125]; this suggests that it inhibits not just by chelation of Fe(II) in solution, but by binding at the active site, in competition with 2OG. Finally it is notable that various potential and actual metabolites of aspirin inhibit the PHDs and cause HIF upregulation in human cells,[126] an observation that may be of interest with respect to the prophylactic effects of aspirin.

No crystal structures have yet been reported for catechol or flavonoid inhibitors in complex with 2OG oxygenases, and only in selected cases have they been shown to be competitive with substrate or cosubstrate binding. Structures have, however, been reported for anthocyanidin synthase in complex with various flavonoids, which may provide some indication of how flavonoids act as inhibitors. Notably, in one case two flavonoid molecules were observed to bind at the active site.[127]

7. Inhibition by metal ions

Inhibition of 2OG oxygenases by divalent transition metal ions has been extensively observed, and because of the relatively weak binding of the Fe ion at the active site, is likely to be a general feature. The inhibition of C-P4H, PLOD1, PHD1-3, FIH, TauD, ABH2, JMJD1A and JMJD2A/E by divalent transition metal ions has been reported, with Mn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Zn^{2+} generally being found to be the most potent inhibitors.[128–133] In most cases, inhibition is attributed to simple competition with Fe^{2+} for binding to the active site chelating residues, though in some cases binding at non-active site location(s) may be relevant.[134] The varying affinities of Fe^{2+} for the 2OG oxygenases are probably reflected in the degree to which those enzymes are inhibited by transition metals. Co^{2+} treatment of cells is used to mimic cellular hypoxia, probably in part by inhibition of the prolyl hydroxylases though other mechanisms cannot be ruled out. Similarly, several metals have been shown to affect global histone and DNA methylation levels, presumably, at least in part, by inhibition of some or all of the 2OG dependent histone demethylases (reviewed in ref. 135).

There is evidence from both *in vivo* and *in vitro* experiments that suggests that a different mechanism may sometimes operate for the inhibition of some 2OG oxygenases by Co^{2+} and Ni^{2+}.[136] These metal ions, and some others, are capable of degrading ascorbate under certain conditions. Thus Ni^{2+} and Co^{2+} provide an alternative contribution to inhibition of enzyme activity, *viz*. degradation of the sometimes necessary cofactor, ascorbate. However, the degree to which different mechanisms for metal inhibition operate, including competition for active site binding and ascorbate degradation, will probably depend on the relative localised concentrations of enzyme, metals and reducing agents (among other factors) in cells.

Overall, the available evidence suggests that all 2OG oxygenases will probably be inhibited to some degree by some transition metals. Hence, proposals that the effects of transition metals on one particular subclass of oxygenases might be usefully exploited therapeutically[137] need to consider the effects of inhibition of other 2OG oxygenases and other metalloenzymes. Nonetheless it is notable that Co^{2+} induces erythropoiesis and has been used in the past as a treatment for anaemia.[138]

8. Inhibition by prime substrate competitors

Many reported potent inhibitors of 2OG oxygenases likely compete with prime substrate as well as with 2OG. However, there are few examples of the inhibitors that compete solely with the prime substrates. Peptide-based substrate analogues have been shown to inhibit the collagen prolyl and lysyl hydroxylases, and the HIF prolyl-hydroxylases. Hydroxylysine-rich peptides inhibited PLOD1,[123] while polyproline was shown to inhibit C-P4H.[139] Peptides containing the non-physiological amino acids 5-oxaproline and 3,4-dehydroproline are substrate analogue inhibitors of C-P4H *in vitro* and in cells.[140–142] HIF-α peptide fragments, where hydroxylated prolines were replaced by alanine residues, apparently inhibit the PHDs in cellular assays.[143] FIH is inhibited by the protein Mint3 (also known as amyloid β-precursor protein-binding, family A), which may compete with the HIF primary substrate for binding (see below).[144] Finally, the availability of substrate–enzyme complex crystal structures for several human 2OG oxygenases (including FIH, PHD2, JMJD2A, PHF8 and ABH2/3)[18,23,26,67,145,146] suggests that the rational design of substrate-analogue inhibitors, whether peptide-based or not, should now be possible.

The only reported instance of a non-peptide substrate analogue acting as a useful inhibitor of a human 2OG oxygenase is the inhibition of γ-butyrobetaine hydroxylase by 3-(2,2,2-trimethylhydrazinium)propionate (THP), also known as Mildronate/MET88. The mechanism of action of THP is described below. Data for the inhibition of other 2OG oxygenases by THP have not been reported. However, given the structural similarity of γ-butyrobetaine to the trimethyllysyl substrates of several histone demethylases (particularly the JMJD2, JMJD3 and JARID1 families of demethylases), the potential effects of THP in inhibiting these demethylases cannot be ruled out.

9. Endogenous inhibitors of 2OG oxygenases

To date there has been relatively little work on the identification of specific 'endogenous' inhibitors that regulate the activity of human 2OG oxygenases. However, in studies on cancer metabolism there is considerable interest in the extent to which tricarboxylic acid (TCA) cycle intermediates and related metabolites inhibit 2OG oxygenases.[147] This is because many tumours possess mutations that impair the activity of TCA cycle enzymes, including to the genes encoding for succinate

dehydrogenase, fumarate hydratase (fumarase) and isocitrate dehydrogenase (IDH).[148–153] Mutations to these enzymes can result in elevated levels of succinate (which is also a coproduct of 2OG oxygenase catalysis) and fumarate, which may result in inhibition of 2OG oxygenases. Point mutations of isocitrate dehydrogenase are particularly interesting because they result in blockage of 2OG production and a gain of function activity, in that the mutant enzymes produce 2-(*R*)-2-hydroxyglutarate ((*R*)-2HG),[151–153] which may also act as a 2OG oxygenase inhibitor. The mutations in the *IDH* gene strongly correlate with the incidence of brain tumours. Interest in the *IDH* mutations is particularly strong because mutations in the dehydrogenases that interconverts both (*R*)- and (*S*)-2HG with 2OG also have severe pathophysiological effects, including, in the case of the (*S*)-2HG dehydrogenase, links with brain tumours.[154,155]

Although systematic studies have not been reported, various studies have shown that succinate, fumarate and other TCA cycle intermediates do inhibit 2OG oxygenases with varying degrees of potency with IC_{50} values ranging from the micromolar to millimolar range. Succinate is a competitive inhibitor with respect to 2OG[123,156] that binds to Fe(II) in the active site of the enzyme (for examples, see the structure of JMJD2A·Ni(II)·succinate, PDB ID 2Q8D[157] and of FIH·Fe(II)·succinate, PDB ID 2CGN), and has been shown to inhibit C-P4H, PLOD1, PHD1-3, FIH and JMJD2E, with varying degrees of potency (Table 7).[91,123,148,156,158,159] The PHDs display the strongest reported inhibition by succinate, whereas FIH and the histone demethylase JMJD2E are only weakly inhibited.

Fumarate inhibits the HIF prolyl hydroxylases PHD1-3 (IC_{50} = 20–80 μM)[38,158,159] but, like succinate, is a relatively weaker inhibitor of FIH (a crystal structure is reported for the FIH·Fe(II)·fumarate complex, PDB ID 2CGO),[158] suggesting that any pathophysiological effects of succinate dehydrogenase and fumarate hydratase mutations are more likely due to inhibition of the HIF prolyl hydroxylases than of FIH. However, further cell-based work is required, and the extent to which inhibition of the 2OG oxygenases in cells by succinate and fumarate is pathophysiologically relevant, is presently unclear.

Other TCA cycle intermediates have been reported to display varying degrees of inhibition of 2OG oxygenases. The lysyl hydroxylase PLOD1 is most potently inhibited by oxaloacetate and citrate, though competitive inhibition was also demonstrated for pyruvate.[123] Similarly, oxaloacetate is a relatively potent inhibitor of both C-P4H and C-P3H, in contrast to pyruvate, which inhibits the collagen prolyl hydroxylases very weakly.[160] FIH and PHD3 are strongly inhibited by citrate. All other TCA cycle intermediates tested against the HIF hydroxylases (pyruvate, oxaloacetate, isocitrate and malate) displayed only weak inhibition, or did not inhibit at all.[158] We have also found that both (*R*)- and (*S*)-2HG inhibit human 2OG oxygenases with a range of potencies.[161]

Although care should be taken in the interpretation of the biological relevance of these results, the available data suggest that, at the highly elevated levels of these metabolites observed in some tumours (>10 mM), accumulation of TCA cycle intermediates may inhibit some 2OG oxygenases in a functionally relevant manner. One possibility is that inhibition of the HIF hydroxylases by TCA cycle intermediates (or 2-hydroxyglutarate) results in elevated HIF activity, upregulating vascular endothelial growth factor (VEGF), resulting in angiogenesis that enables tumour growth.[148] However, further work is required to test how selective the elevated levels of TCA cycle intermediates are in terms of 2OG oxygenase inhibition.

A further line of investigation has concerned the modulation of 2OG oxygenase activity by proteins, with one focus being on the HIF hydroxylase FIH. In addition to hydroxylation of the HIF-α subunit, FIH also accepts many ankyrin repeat domains including IκBα, Notch, *etc*., as substrates and likely interacts with ankyrin repeats that do not act as substrates,[162–169] but which may act as inhibitors. Collectively, it is proposed that the interaction of FIH with multiple ankyrin repeat domains (>100) serves to modulate the role of FIH in the hypoxic response, including by altering the range over which the HIF hydroxylases act as sensors in the hypoxic response.[170] A recent report has also identified a non-ankyrin protein inhibitor of FIH, Mint3; however, its mechanism of action is unknown.[144]

10. Towards the therapeutic inhibition of 2OG oxygenases

Several human 2OG oxygenases have been investigated as potential therapeutic targets. In this section, we focus on some of the known human and plant enzymes, the rationale behind the choice of these 2OG oxygenases as targets for small-molecule inhibitors, and the efforts towards development of selective inhibitors.

Table 7 Table of available inhibition data for TCA cycle intermediates as inhibitors of 2OG oxygenases. IC_{50} or K_i values are shown, with K_i values in parentheses. See Table 1 for definition of the 2OG oxygenases

	C-P4H	C-P3H	PLOD1	PHD1	PHD2	PHD3	FIH	JMJD2E
Succinate	(1.5 mM)[156]	(0.8 mM)[160]	(5–15 mM)[123]	(350 μM)[159]	(460 μM)[148,159] 85 μM[38] 19 μM[158]	(430 μM)[159]	(>10 mM)[159]	320 μM[68]
Pyruvate	(15 mM)[160]	(4.2 mM)[160]	(10–20 mM)[123]	(350 μM)[159]	(350 μM)[159]	(350 μM)[159]	(>10 mM)[159]	
Oxaloacetate	(0.1 mM)[160]	(0.5 mM)[160]	(0.4–0.6 mM)[123]	(400 μM)[159]	(1 mM)[159]	(590 μM)[159]	(400 μM)[159]	
Citrate			0.3 mM[123]		(1.8 mM)[159]	(180 μM)[159]	(110 μM)[159]	
Isocitrate			2 mM[123]	(350 μM)[159]	(350 μM)[159] 41 μM[158]	(350 μM)[159]	(5 mM)[159]	
Malate			1 mM[123]	(>10 mM)[159]	(>10 mM)[159]	(>10 mM)[159]	(>10 mM)[159]	
Fumarate			2 mM[123]	(80 μM)[159]	60 μM[158,159] 19 μM[38] 3 μM[158]	(50 μM)[159]	(>10 mM)[159]	2.3 mM[68]

10.1 Collagen prolyl hydroxylases

Several 2OG oxygenases are involved in the biosynthesis of collagen, which forms the bulk of the extracellular matrix.[3,171] Pathological fibrosis of organs including the liver, kidneys, lungs and skin is characterised by excessive accumulation of the extracellular matrix; in the case of liver fibrosis, this is often in response to chronic inflammation as a result of alcohol-induced liver damage, or as a result of liver infections such as hepatitis C.[171] Pulmonary fibroses can form in response to irritant inhalation, while kidney fibrosis is characteristic of renal disease. Therapeutic intervention to prevent the progression of fibrosis is thus desirable. This intervention may take place at the inflammatory stage of fibrosis, at the stage where fibroblasts are recruited and activated, or at the final stage, the excess deposition of the extracellular matrix by fibroblasts.[171]

A primary component of the extracellular matrix is fibrillar collagen, which is composed of a triple helix of three separate collagen strands. The stability of this triple helix substantially relies on 4-hydroxylation of prolyl residues in the canonical -Xaa-Yaa-Gly- motif, where Xaa is proline, and Yaa is proline or lysine. Prolyl-4-hydroxylation at the Yaa position is catalysed by the 2OG dependent collagen prolyl-4-hydroxylase (C-P4H, Fig. 2b), and stabilises the collagen triple helix fold. Reduction in the 4-hydroxyproline content of collagen leads to significant decreases in its structural stability, renders it vulnerable to proteolytic cleavage, and impairs its ability to interact with other components of the extracellular matrix.[172] Thus C-P4H has been targeted for therapeutic intervention; the possibility of reducing extracellular matrix deposition by inhibiting the collagen prolyl-4-hydroxylase has been investigated extensively, with some compounds progressing to clinical trials (see below); however, to date no drugs have been approved for clinical use.[9,171] As the treatment of fibrosis likely requires long-term inhibition of C-P4H, highly selective inhibitors that do not inhibit other 2OG oxygenases may be required for clinical application.[9] It is also notable that prolyl and lysyl residues are hydroxylated in 'collagen-like domains' in non-collagen proteins.

Collagen is also hydroxylated at prolyl C-3 positions and at lysyl C-5 positions (Table 1, Fig. 2b). Prolyl-3-hydroxylation by collagen prolyl-3-hydroxylases is catalysed by C-P3H/LEPRE;[173] hydroxylation at this position is proposed to destabilise the collagen fold, but to differing extents depending on whether 3-hydroxyproline occurs at position Xaa or Yaa in the collagen amino acid sequence.[174,175] Hydroxylation of collagen lysyl residues at the C-5 position by PLOD enzymes acts as an anchor point for attachment of galactose or glucosylgalactose, which form crosslinks to other collagen strands.[3,176] However, mutations to enzymes catalysing these modifications are linked to diseases (mutations in the collagen lysyl hydroxylases result in Ehlers–Danlos syndrome type VI, an inherited connective tissue disorder (reviewed in),[177] and mutations in the collagen prolyl-3-hydroxylase LEPRE1 are a cause of recessive osteogenesis imperfecta).[178]

Early studies on the *in vitro* inhibition of C-P4H identified 2,2′-bipyridine and succinic acid as inhibitors.[156] During the course of kinetic studies, it became apparent that a wide range of 2OG analogues were competitive inhibitors of C-P4H, including pyridine-2-carboxylates (with 2,5-pyridinedicarboxylate (2,5-PDCA) being the most potent identified inhibitor), catechols and anthraquinones, all of which likely inhibit by competing with 2OG for binding to the active-site Fe(II) (Fig. 5).[42,124,179,180] Importantly for these early studies, many of these compounds were found to be selective for inhibition of C-P4H over 2OG dehydrogenase, which does not employ an active site metal.[160]

Efforts aimed at developing selective inhibitors of C-P4H for the treatment of fibrosis then focused on two compound series (Fig. 7): derivatives of 2,5-PDCA and derivatives of *N*-oxalylglycine (which were subsequently found to be generic inhibitors of 2OG oxygenases (see above)). It was shown for pyridine carboxylates that the ability to chelate active-site Fe(II) *via* the pyridinyl nitrogen and the 2-substituent was necessary for potency (though pyridine-2-carboxylic acid, unlike 2,4-PDCA or 2,5-PDCA, was not a very potent inhibitor of C-P4H).[181] Variation in the 2-substituent (replacing the carboxylate with oximes and nitrogen heterocycles) led to marginal increases in potency, with the exception of 2-(3-methyl-4*H*-pyrazol-4-onyl)-pyridine, which had an IC_{50} = 20 μM, and 2-(acetylaceto)-pyridine, with an IC_{50} = 9 μM.[181] Extension of the distance between the pyridine-carboxylate ring and the 5-carboxylic acid had little effect on potency of inhibition of C-P4H.[182] Further efforts demonstrated that a wide degree of variability in the 5-position was possible (Fig. 7),[181–185] suggesting that these compounds were probably not interacting with the basic 2OG-anchoring residue in the active site.[182] Support for this proposal was provided by the fact that pyridine-2-carboxylate-5-amides were as potent as the parent compound, pyridine-2,5-dicarboxylate. 5-Acylsulfonamides (including phenyl, naphthyl and quinolyl acylsulfonamides) were even more potent,[182] suggesting that the 'turn' in the side chain induced by the sulfonamide might aid inhibitor binding.[184]

2,2′-Bipyridine, a bidentate iron chelator, is a potent inhibitor of C-P4H; as for other 2OG oxygenases, its effects may in part be due to chelation of Fe(II) in solution. 2,2′-Bipyridine-5,5′-dicarboxylic acid is a much more potent inhibitor than bipyridine itself, with an IC_{50} value significantly lower than the solution concentration of Fe(II) under the reported assay conditions, thus suggesting that it inhibits by binding to the active site.[183] The related compound 4-oxo-1,10-phenanthroline-3-carboxylate (FG-0041, Fig. 7) was designed as an inhibitor of C-P4H (which it inhibited *in vitro* and in cell culture),[185] but was later also demonstrated to inhibit the HIF prolyl hydroxylases[186] and FIH (as were derivatives of FG-0041);[187] thus, further chemical modifications of these general bipyridyl templates are required to achieve selectivity.

In contrast, while *N*-oxalylglycine (and *N*-oxalyl-(*S*)-alanine) inhibit C-P4H, attempts to modify the *N*-oxalylglycine template resulted in compounds that were less active, *e.g.* *N*-oxalo-β-alanine, suggesting that *N*-oxalyl amino acids inhibit by binding both to the active-site metal and to the distal 2OG-anchoring residues.[86,87] Substitutions at the C-α position did not lead to increases in potency (Fig. 7), though it was observed that some compounds were more active than their enantiomers, indicating some stereoselectivity of inhibition

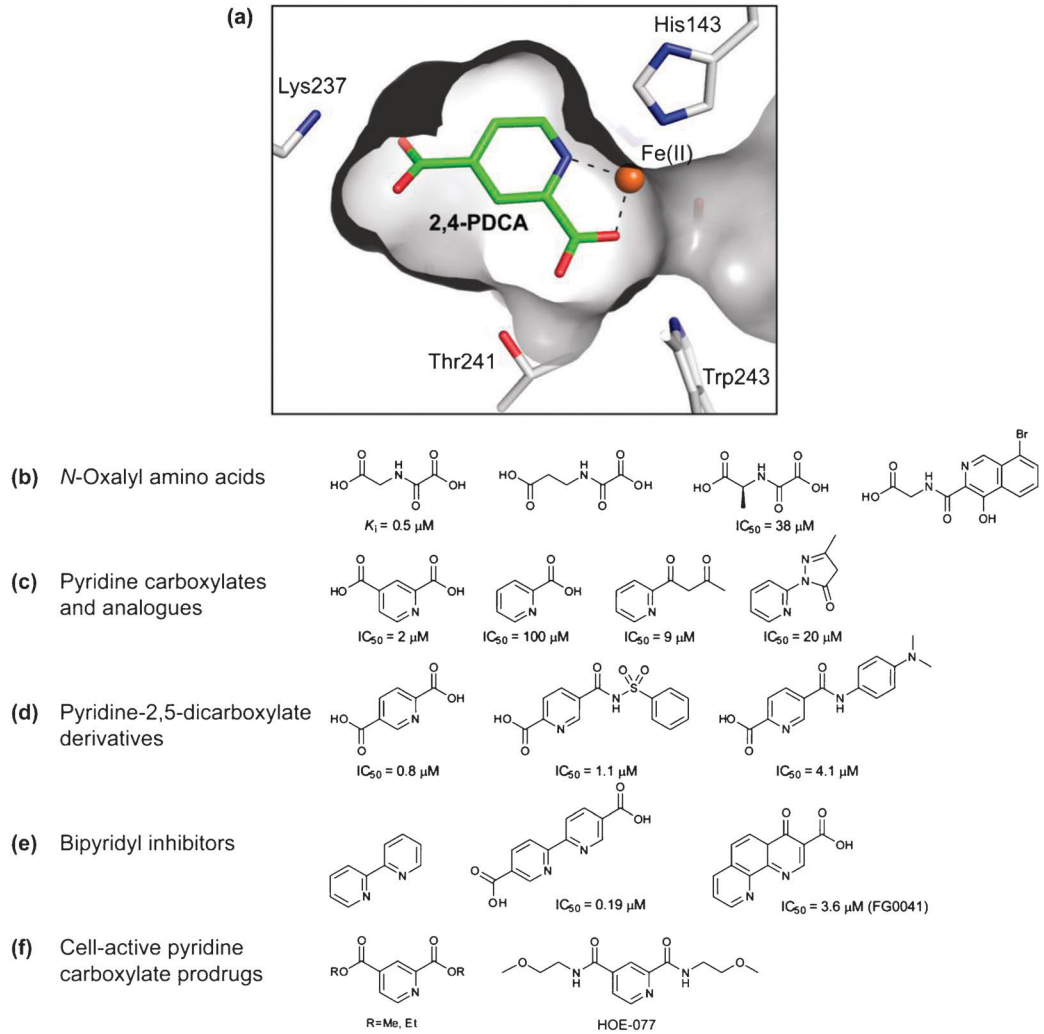

Fig. 7 Development of inhibitors of collagen prolyl-4-hydroxylase as potential antifibrotic agents. (a) View from a crystal structure of a collagen prolyl-4-hydroxylase (C-P4H) from *C. reinhardtii* in complex with 2,4-PDCA (PDB ID 2JIG), (b) *N*-oxalyl amino acid C-P4H inhibitors; while *N*-oxalylglycine and *N*-oxalyl-L-alanine were potent inhibitors, *N*-oxalyl-β-alanine was significantly less potent. Bicyclic analogues of *N*-oxalylglycine were also developed as inhibitors. (c) 2,4-Pyridinedicarboxylate and analogues of the 2-carboxylate were potent inhibitors, while pyridine-2-carboxylate (without the 4-carboxylate) was a weak inhibitor. (d) 2,5-PDCA was among the most potent C-P4H inhibitor characterised; a variety of substituents were tolerated at the C-5 carboxylate, suggesting an alternative mode of binding to that shown for 2,4 PDCA in (a) (*i.e.* the C-5 carboxylate does not interact with Lys237, as observed for 2,4-PDCA). (e) Bipyridyl inhibitors of C-P4H. (f) Cell-active forms of 2,4-PDCA used in preclinical experiments. Reduction of hepatic fibrosis was achieved with HOE-077, but the mechanism of action may not be solely *via* inhibition of C-P4H.

within the active site (see above). Replacement of the *N*-oxalyl carbonyl group of *N*-oxalylglycine with an isoquinoline moiety gave compounds with good activity against C-P4H *in vitro* and *in vivo*.[110] This particular strategy (replacement of the oxalyl group with a suitable metal-chelating heterocycle) found extensive use more than a decade later in the design of HIF prolyl hydroxylase inhibitors (see below).

Crystal structures have not yet been reported for any of the animal enzymes involved in collagen hydroxylation (though structures of an algal C-P4H from *Chlamydomonas reinhardtii* have been determined; see Fig. 7).[107,188] In particular, difficulties in isolating the catalytic subunit of C-P4H (which exists as a heterotetramer with protein disulfide isomerase) have contributed to the lack of progress in crystallographic efforts. The lack of structural information has probably hindered the development of more selective inhibitors of the C-P4H and the HIF prolyl hydroxylases.

Most C-P4H inhibitors that were potent inhibitors *in vitro* were either not tested in cells, or were inactive; further, compounds that were active in cells were prodrug forms of *in vitro* inhibitors such as 2,4-PDCA (where a variety of ester[189] and amide[190,191] derivatives were evaluated and found to be much more active than 2,4-pyridinedicarboxylic acid in cells),[179] *N*-oxalylglycine (where the dimethyl ester inhibited C-P4H activity in cell culture)[86] or 3,4-dihydroxybenzoate, where the ethyl ester was active in cells.[74] One derivative of 2,4-PDCA, HOE-077 (pyridine-2,4-(bis(2-methoxyethyl))-carboxamide, Fig. 7), was tested in rats and dogs in preclinical trials, where it was found to inhibit collagen synthesis in the liver. However, this prodrug form was not observed to be

metabolised to 2,4-PDCA in either of these animal models, though there was some evidence that some of the metabolites were able to inhibit C-P4H activity.[191] Further studies indicated that HOE-077 may inhibit collagen synthesis by inactivating hepatic stellate cell formation (collagen synthesis occurs in hepatic stellate cells in fibrosis), rather than by direct inhibition of C-P4H.[192]

Although these studies showed some activity of prolyl hydroxylase inhibitors in cells, the off-target effects (these compounds were also shown to inhibit lysyl hydroxylase and γ-butyrobetaine hydroxylase),[193,194] uncertainties over the mechanism of action (it was unclear whether the expected metabolites were actually formed, and whether inhibition of C-P4H was the primary mode of action), and the requirements for long-term dosing to prevent recurrence of collagen synthesis and deposition in fibrosis has meant that, to date, efforts to develop therapeutically useful C-P4H inhibitors have been unsuccessful. Interestingly, in studies with rats, one C-P4H inhibitor (FG-0041) was shown to alter left ventricular enlargement and to assist in recovery of left ventricular function after myocardial infarction. It is possible that this effect is mediated *via* HIF hydroxylase inhibition (see Section 10.4).[195] Since the initial studies on inhibition of the collagen hydroxylases, however, significant advances have been made in our understanding of the roles and structures of other human 2OG oxygenases and it may be that new efforts to identify selective and therapeutically useful inhibitors may be fruitful.

10.2 Gibberellin oxidases

Plant growth retardants (PGRs) are used in agriculture to alter plant morphology by reducing shoot growth, *via* a lowered rate of cell division and a reduction in cell elongation.[196] Limiting plant growth has a number of advantages, such as preventing damage in cereal crops, curbing unwanted vegetative growth, improving the ratio of vegetative growth to fruit production[197] or reducing the size of ornamental plants.[198] Most PGRs that are used in agriculture or horticulture act in an antagonistic manner to the gibberellin (GA) and auxin plant hormones. Gibberellins play signalling roles in responses to temperature, stress and light and are involved in the control of leaf expansion, seed germination and flowering time (reviewed in ref. 199).

Many PGRs exert their effects primarily by interfering with gibberellin biosynthesis, and can be classified according to the stage at which they interact with this biosynthetic pathway.[197] More than 100 diterpenoid gibberellic acids (GAs) have been identified though only a small number are active phytohormones, while the non-active GAs are likely to be biosynthetic intermediates or deactivated metabolites.[199] Gibberellin biosynthesis begins with geranylgeranyl diphosphate, which is converted by terpene synthases and cytochrome P450 monoxygenases to give a key intermediate, GA_{12}, that is subsequently converted to bioactive GAs through reactions catalysed by the 2OG oxygenases GA C20-oxidase and GA C3-oxidase/GA 3β-hydroxylase (Fig. 8a).[103,199,200] Deactivation of GAs can be mediated by the action of the 2OG-dependent GA 2-oxidases, which regulate levels of bioactive hormones by C-2 hydroxylation.[201] Gibberellin C20-oxidase is a 2OG dependent oxygenase catalysing sequential oxidations at the C-20 methyl group of the GA molecule.[202]

Various inhibitors, all likely 2OG competitors, and most notably prohexadione, trinexapac-ethyl and daminozide inhibit the 2OG oxygenases involved in gibberellin biosynthesis, and have been used in agriculture as growth retardants, with applications in stem stabilisation of a variety of crops, including cereals, rapeseed and rice (reviewed in ref. 197). Prohexadione and trinexapac-ethyl (Fig. 8b) are structural mimics of 2OG that have been shown to compete with 2OG for binding to GA C3-oxidase/GA 3β-hydroxylase.[203] Interestingly, replacement of the cyclohexadione ring with related aromatic rings ablated activity towards the gibberellin oxidases.[204] Esters or calcium salts of prohexadione and trinexapac-ethyl have found widespread application as growth retardants for many different plant species.[197]

The small molecule daminozide, or succinyl *N,N*-dimethyl hydrazide, was identified, together with maleyl *N,N*-dimethyl hydrazide (Fig. 8b), as a growth retardant when applied to plant foliage.[205] The analogous phthalic acid derivatives were inactive as growth retardants. The maleic acid derivative was later shown to decompose in aqueous solution by intra-molecular reaction,[206] leading to the subsequent use of the succinic acid derivative, daminozide, in agricultural applications. The mechanism of action of daminozide was later shown to be, at least in part, inhibition of gibberellin biosynthesis as demonstrated by the ability of the compound to inhibit 3β-hydroxylation of GA_9 to GA_4 in cell free extracts.[207] Daminozide was used in agriculture, particularly to control pre-harvest ripening of apples, until concerns about its possible carcinogenicity led to its withdrawal from use, though it continues to find use in the cultivation of ornamental plants.[208–210] Prohexadione and daminozide also inhibit flavanone 3β-hydroxylase, and likely inhibit other 2OG oxygenases as well.[109]

10.3 Ethylene biosynthetic enzymes

Ethylene is an important and widely distributed signalling molecule in plants, and is involved in many developmental processes including senescence and fruit ripening.[211] Ethylene is biosynthesised from *S*-adenosyl-L-methionine (*S*-AdoMet) first by conversion into 1-amino-1-cyclopropane-1-carboxylate (ACC) which is oxidised by ACC oxidase to form ethylene, CO_2 and HCN (Fig. 9).[211–213] In addition to its essential oxygen cosubstrate, ACC oxidase is also strongly dependent on bicarbonate (or CO_2)[214] and ascorbate[215] for activity. Although it does not use 2OG, the structure of ACC oxidase reveals it as being closely related to the 2OG oxygenases.[216] Small molecule inhibitors of ACC oxidase have not been extensively used; however, it is known to be inhibited by substrate analogues and some generic 2OG oxygenase inhibitors, *e.g.* Co(II). Ethylene biosynthesis is widely targeted for control of soft fruit (*e.g.* tomatoes) ripening by genetic intervention (see ref. 217 for review). Several of the small molecules used as GA biosynthesis inhibitors also inhibit the ethylene-forming enzyme, ACC oxidase,[216] thus having the additional effect of controlling fruit ripening.[218] The demonstration that ACC

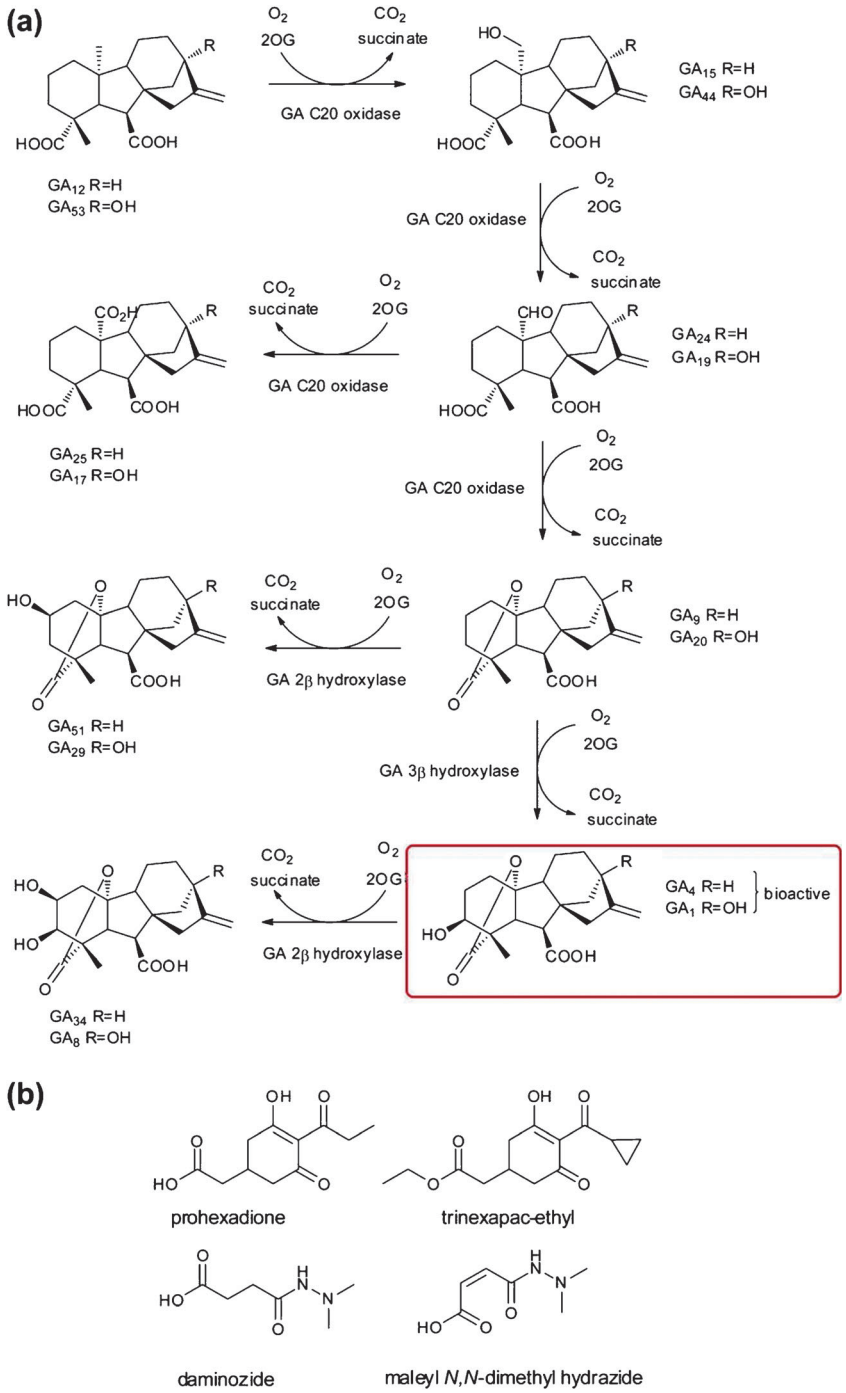

Fig. 8 Role of 2OG oxygenases in the biosynthesis of plant signalling molecules. (a) Steps in gibberellin synthesis. Starting with GA_{12} or its C-13 hydroxylated analogue GA_{53}, sequential oxidations at position C-20 lead to formation of alcohol (GA_{15}/GA_{44}) then aldehyde (GA_{24}/GA_{19}) intermediates before formation of either a carboxylic acid (GA_{25}/GA_{17}) or loss of C-20 and formation of a lactone bridge (GA_9/GA_{20}). Subsequent conversion to biologically active gibberellins is by the action of GA 3-oxidase/GA 3β-hydroxylase enzymes. (b) Commercially used plant growth regulators.

oxidase is inhibited by 2-oxo acids supports links with the 2OG oxygenases.[219]

10.4 Inhibition of γ-butyrobetaine hydroxylase (BBOX1)

Carnitine (L-3-hydroxy-4-*N,N,N*-trimethylaminobutyrate) plays an essential metabolic role by enabling transport of long chain fatty acids into the mitochondria of animals, plants and microorganisms.[220] Other roles of carnitine include transport of the products of α-oxidation, fatty acid storage, excretion of toxic products, and maintaining acyl-coenzyme-A/coenzyme-A homeostasis.[220] Carnitine can be obtained from the diet and/or by biosynthesis in animals, and is commonly used as a dietary supplement as it is proposed to promote fatty acid

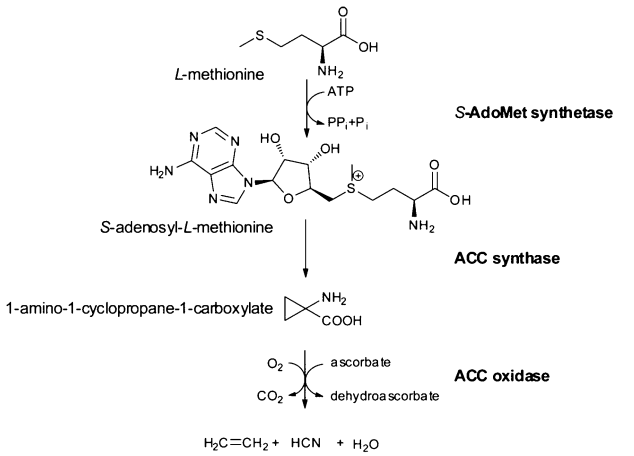

Fig. 9 Steps in the biosynthesis of ethylene. The final step is the ascorbate-dependent conversion of ACC to ethylene which also leads to formation of water and hydrogen cyanide. ACC oxidase activity is stimulated by bicarbonate/CO_2.

metabolism. In animals, the starting point for carnitine biosynthesis is N^{ε}-trimethyllysine, which likely originates from the hydrolysis of N^{ε}-methylated proteins (Fig. 10). Two steps of carnitine biosynthesis are catalysed by Fe(II) and 2OG dependent oxygenases: N^{ε}-trimethyllysine hydroxylase (TMLH) and γ-butyrobetaine (γBB) hydroxylase (BBOX1).[221–224] It is proposed that inhibition of carnitine biosynthesis will reduce fatty acid oxidation and, in doing so, reduce the rate at which reactive oxidising species are produced. Although the basis for the mechanism is uncertain, this is proposed to be beneficial for patients after cardiac arrest or myocardial infarction.[10]

BBOX1 is the proposed target of therapeutic inhibition by 3-(2,2,2-trimethylhydrazine)propionate (THP/Mildronate/MET88), which is clinically used as a cardioprotective treatment after myocardial infarction.[10,225] JSC Grindeks, the company that markets THP, also suggests that it acts to ameliorate blood flow restriction in the ischemic region during acute and chronic ischemic cerebral vascular disturbances. THP is promoted for uses in cases of decreased physical performance, physical and psychoemotional overload, and during recovery processes, though a mechanism of action is not provided for these indications. It has been reported that successful clinical trials for the use of THP for the treatment of angina have been completed. Other studies suggest that THP may be beneficial for the treatment of seizures and alcohol intoxication, though the mechanism of action is unclear.[226]

Recent biochemical and structural studies of human BBOX1[17,227] in complex with substrates and the inhibitors *N*-oxalylglycine and THP (Fig. 11) show that THP is also a substrate for BBOX1, reacting to give several products, including formaldehyde, dimethylamine, *tert*-butylamine, malonic acid semialdehyde, and 3-amino-4-(*N*-methyl-*N*-hydroxy-methyl)-aminobutanoic acid.[17] These observations may have important implications for the clinical use of THP, because the products of inhibitor degradation may have undesirable properties.

The BBOX1 crystal structure provides a basis from which a structure guided inhibitor design can proceed and will aid in the development of more selective inhibitors. It also reveals close similarity between BBOX1 and TMLH, suggesting that THP will likely also inhibit TMLH. The structure also shows that BBOX1 forms a homodimer using its unique *N*-terminal Zn-binding domain. Because the Zn-binding site is close to the active site opening, it may be possible to inhibit BBOX1 using Zn-ejectors, as has been demonstrated for the JMJD2 family of histone demethylases.[27]

Recent patent applications have described synergistic properties of the fumarate, succinate and orotate salts of THP (WO2009074498; US20060264506). Because fumarate, succinate and orotate are 2OG analogues which inhibit a range of 2OG oxygenases, it is possible that the synergistic effects may be due to dual inhibition of BBOX1 by both 2OG and γBB mimetics or, possibly, that off-target inhibition of other 2OG oxygenases such as the HIF hydroxylases is contributing to the effects observed.[148–150,158]

10.5 Inhibition of the HIF hydroxylases

The physiological effects of hypoxia have been studied for over a century. However, it is only relatively recently that the molecular details of the hypoxic response system (Fig. 12) have become apparent. A major breakthrough came with the discovery of the α,β-heterodimeric hypoxia inducible factor (HIF) by Semenza and coworkers in studies on the mechanism by which erythropoietin (EPO) is regulated. EPO regulates red blood cell production, and recombinant EPO is widely used for the treatment of anaemia. It was shown that α,β-HIF binds to 'hypoxia response elements' (HREs) associated with the EPO gene, so increasing its rate of transcription. Importantly, it was found that the levels of the HIF-α subunit increased in hypoxia.[84,228]

Subsequently it was found that binding of HIF-α to the von Hippel–Lindau (VHL) protein increases with rising oxygen levels, and that the von Hippel–Lindau protein acts as a targeting component for a ubiquitin E3 ligase, so signalling for the degradation of HIF-α *via* the proteasome.[229] The modifications that triggered HIF-α binding to the VHL protein were shown to be prolyl-hydroxylation of two prolyl residues in the 'oxygen-dependent degradation domain' of HIF-α.[230,231] The oxygen of the hydroxyprolyl residue was shown to be derived from molecular oxygen,[232] providing a direct link between transcriptional regulation and physiological oxygen levels. Significantly, HIF-α prolyl hydroxylation was inhibited by NOG/DMOG,[233] indicating a 2OG oxygenase as the catalyst. The stereochemistry of hydroxylation was also defined as *trans*-4-hydroxy, supporting a link with the collagen prolyl-4-hydroxylases.[31] Subsequently, bioinformatic analyses coupled to experimental studies were used to identify the HIF prolyl hydroxylases (Fig. 12), first in *C. elegans* and then in human cells. Several kinetic studies have correlated the molecular properties of the HIF hydroxylases with their cellular roles.[47,62,89] It is interesting that the rate-limiting step for the prolyl hydroxylase PHD2, proposed as the most important PHD isoform in higher animals, is proposed to be binding/reaction of oxygen.[47] Early studies identified various 2OG analogues, including compounds already shown to be C-P4H inhibitors, as inhibitors of the PHDs.[83,231]

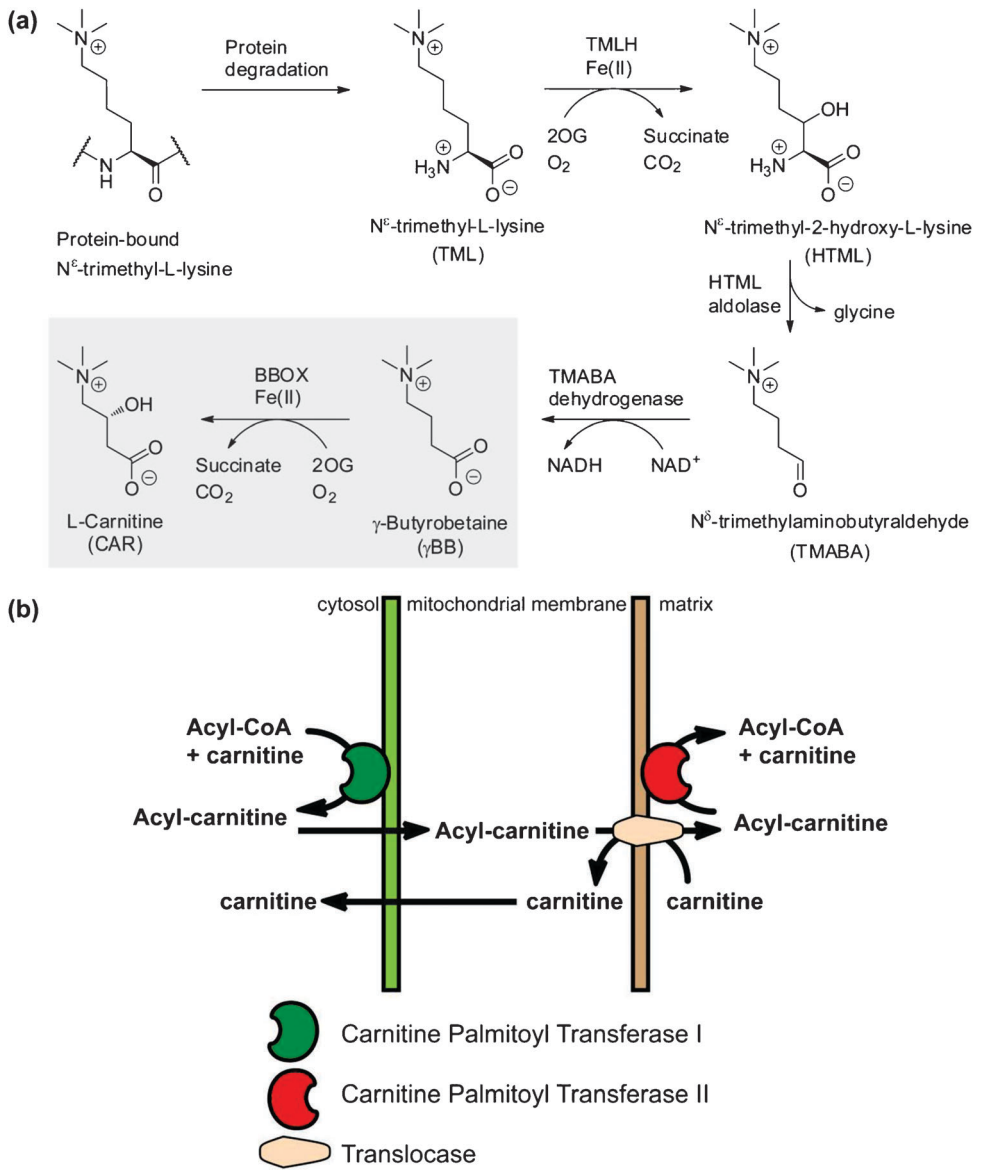

Fig. 10 Role of 2OG oxygenases in carnitine biosynthesis. (a) BBOX and TMLH catalyse steps in carnitine biosynthesis, and (b) function of carnitine/acyl-carnitine in transporting fatty acids across the mitochondrial membrane.

Under normoxia, HIF-α is also hydroxylated at an asparaginyl residue (Asn803 in human HIF-1α) in the C-terminal transcriptional activation domain, in a reaction catalysed by FIH (Fig. 12).[55,228] This hydroxyl group prevents binding of HIF-α to the p300/CBP transcriptional coactivator complex, thus inhibiting HIF-α mediated transcription.

HIF target genes include erythropoietin (EPO), vascular endothelial growth factor (VEGF), and a wide variety of other targets of therapeutic interest (reviewed in ref. 84 and 234). Inhibition of the HIF hydroxylases (PHD1-3 and FIH) is currently being investigated as a means of therapeutic intervention in a variety of conditions associated with hypoxia, including anaemia and ischaemic-related diseases including stroke, *via* modulation of EPO and VEGF levels, respectively (reviewed in ref. 235 and 236).

EPO is a hormone that regulates erythropoiesis (*i.e.* the production of red blood cells). Insufficient EPO is characteristic of anaemia, and recombinant EPO is widely used to treat anaemia. The expression of EPO is regulated primarily by HIF, and thus by the HIF prolyl hydroxylases. Inhibition of the PHDs by small molecules has the effect of stimulating EPO production,[237] and several inhibitors of the PHD enzymes are in clinical trials for the treatment of anaemia (see below).

Angiogenesis is induced by upregulation of VEGF by HIF. In some clinical situations, inducing angiogenesis may be desirable to alleviate the damage caused by ischaemic tissue disease. It has been proposed that ischaemic preconditioning in cerebral and cardiac tissues might be achieved by inhibiting the PHDs/FIH, thus protecting against ischaemic damage as a result of stroke or myocardial infarction.[235] However, angiogenesis assists the growth of oxygen-limited tumours by increasing their blood supply. Thus, any therapeutic inhibition (*e.g.* to treat ischaemia) may have the unwanted effect of promoting tumour growth and metastasis; however, the

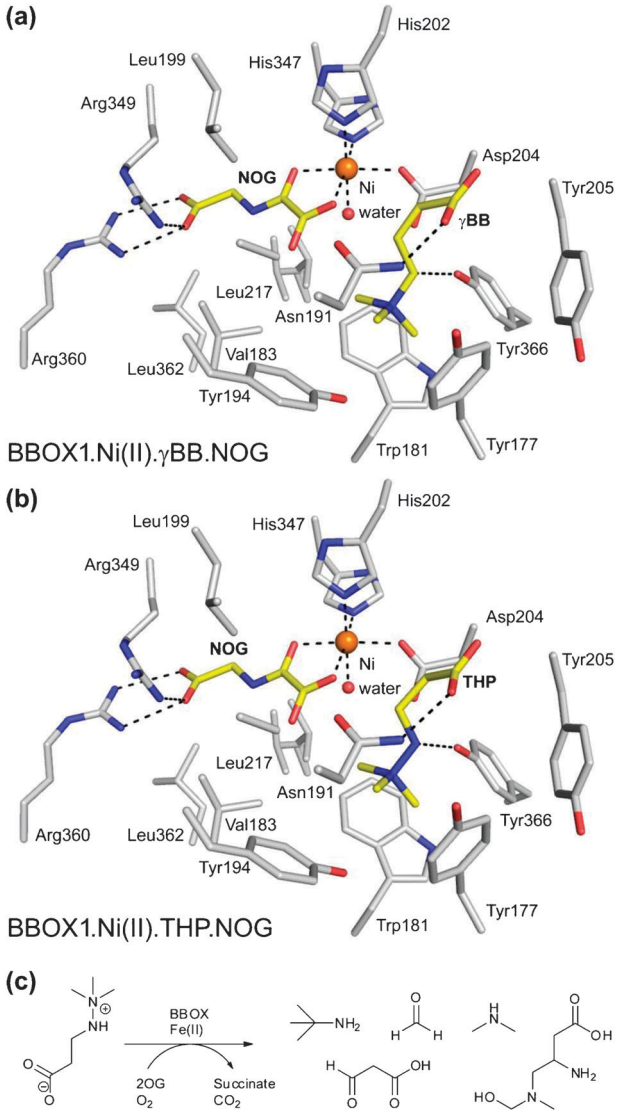

Fig. 11 Views from the crystal structure of human γ-butyrobetaine hydroxylase (BBOX1) in complex with the 2OG analogue NOG and (a) γ-butyrobetaine, or (b) the inhibitor THP. Both γ-butyrobetaine and THP adopt the same binding mode, and both are substrates for BBOX1. In both cases, the trimethylamino moiety is contained within a hydrophobic 'box' similar to that found in many methyllysine binding proteins, such as plant homeodomains, tudor domains, *etc.* (c) The inhibitor THP is a substrate for BBOX1, reacting to form a mixture of products, including formaldehyde, dimethylamine, *tert*-butylamine, malonic acid semialdehyde, and 3-amino-4-(*N*-methyl-*N*-hydroxymethyl)aminobutanoic acid.

available emerging evidence suggests that for PHD inhibition this may not be a problem, at least in the short term. It is also worth noting that improved oxygenation of tumours may also increase their sensitivity to radiotherapy and chemotherapy. Tumours often have abnormal "leaky" blood vessels; hence it is conceivable that HIF activation could further dysregulate this abnormality in a useful manner. However, any therapeutic application involving HIF hydroxylase inhibitors will have to take into account the many hundreds of genes (other than those targeted by the medication) that are also regulated by HIF. Such considerations may be particularly important in the development of inhibitors for diseases such as anaemia, where long term application of inhibitors may be required.

In recent years, extensive medicinal chemistry efforts have been directed towards the development of clinically useful inhibitors of the HIF hydroxylases and in particular the PHDs (recently reviewed in ref. 238). Inhibition of the HIF hydroxylases has significant therapeutic potential, in the treatment of cancer, by rendering tumours more sensitive to radio- and chemotherapy; in the treatment of anaemia, by increasing levels of EPO; and in the treatment or prevention of ischaemic damage, largely by reducing dependence on oxidative metabolism. Three PHD inhibitors have progressed to Phase II clinical trials for the treatment of anaemia,[238] of which the Fibrogen compound FG-2216 (Fig. 13) has demonstrated increased haemoglobin levels in patients.[239] Several other inhibitors are currently in Phase I trials.

Early studies on the HIF prolyl hydroxylases demonstrated that well-characterised inhibitors of the collagen hydroxylases also inhibited the HIF PHDs. *N*-Oxalyl amino acids, including *N*-oxalylglycine, and other 2OG analogues, such as 2-thioglutarate, all inhibited the HIF PHDs.[83,186] Simple hydroxamic acids such as dealanylalahopcin and ciclopirox olamine also inhibited the PHDs. In some cases it was shown that such compounds led to stabilisation of HIF-α, induction of VEGF and stimulation of angiogenesis.[99,100,111] 2,4-Pyridinedicarboxylate inhibited the PHDs, whereas 2,5-PDCA did not,[186] demonstrating that selective inhibition between the two classes of prolyl hydroxylase might be achieved. Some bipyridyl compounds such as FG-0041 (see above) and TM6008 which were designed as C-P4H inhibitors were also shown to inhibit the PHDs.[111,240] Several compounds which combined the pyridine and glycine units were also potent PHD inhibitors[111] (and had previously been reported to inhibit the collagen prolyl hydroxylases,[110] see above).

Crystal structures of PHD2 in complex with isoquinolinyl glycinamide inhibitors have been reported (Fig. 13).[241] Comparison of the PHD2 : bicyclic inhibitor structures with that of the PHD2 : NOG : Mn(II) : HIF-1α fragment structure reveals a conformational change in the β2-β3 loop that folds to enclose the substrate in the active site. In the substrate-bound structure a salt bridge between Asp254 and Arg256 is present; comparison with the PHD2 : bicyclic inhibitor structures reveals that inhibitors likely block this interaction, which suggests that the mechanism of inhibition by the bicyclic inhibitors is more complex than simple competition with 2OG, but also acts by prevention of a conformational change in the enzyme required to bind the substrate in a catalytically productive manner.[38,93]

There are now many reports, both in the academic literature and in patents, of PHD inhibitors based on the glycinamide or related motifs (Fig. 13 and 14). These have recently been reviewed in detail,[238] and we present here a summary of the work, with selected inhibitors shown in Fig. 14. It is important to note that very little (if any) selectivity data have been reported for these compounds as potential inhibitors of any other 2OG oxygenases (with the possible exception of the collagen prolyl hydroxylases). Inhibitors of this type generally employ a glycine motif coupled by an amide bond to an aromatic heterocyclic system. Active-site iron chelation

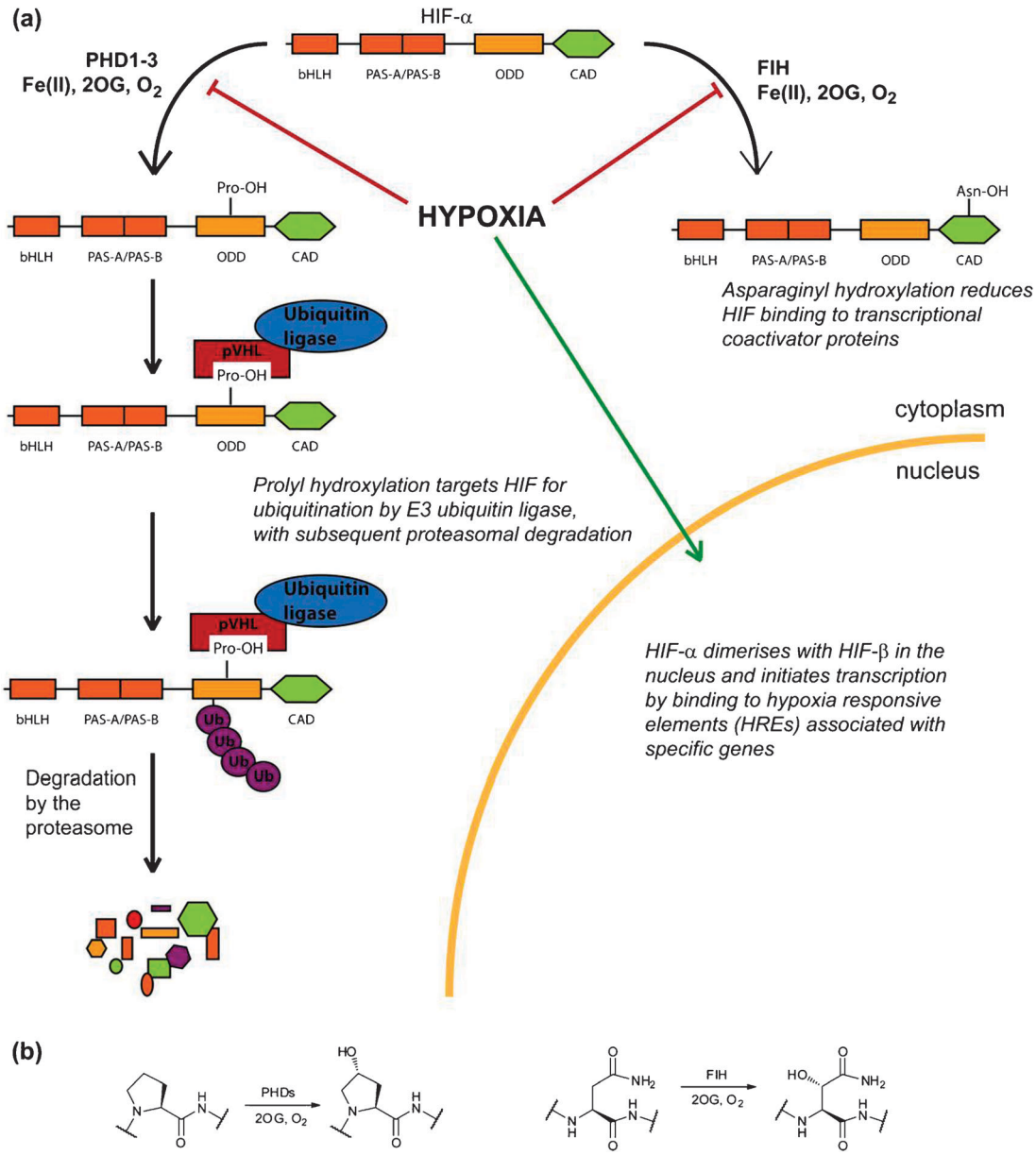

Fig. 12 The role of 2OG oxygenases in the hypoxic response in humans. (a) In humans, three PHD isoforms signal for HIF-α degradation *via* the ubiquitin proteasome system by catalysing HIF-α prolyl hydroxylation. FIH reduces the transcriptional activity of HIF by catalysing asparaginyl hydroxylation of its C-terminal transcriptional activation domain, a post-translational modification that reduces the interaction of HIF with transcriptional coactivator proteins. The bHLH (basic helix–loop–helix) and PAS domains of HIF-α are involved in DNA binding and dimerisation with HIF-β. HIF-α undergoes two prolyl-hydroxylations in its oxygen dependent degradation domain (ODD) and one in its C-terminal transcriptional activation domain (CAD). The von Hippel–Lindau Protein (pVHL) acts as a targeting component for an E3 ubiquitin ligase. (b) Prolyl and asparaginyl hydroxylation reactions catalysed by the PHDs and FIH.

occurs, or is proposed to occur, through the amide carbonyl oxygen and an atom in the heterocycle which is capable of iron chelation. Such heterocycles include pyridines (and variations, such as quinolines, isoquinolines, *etc.*), pyridinones, pyrimidinones and coumarins, indanones and benzimidazoles. Many such heterocycles also include a hydroxyl group adjacent to the glycinamide, which can form a hydrogen bond with a tyrosine side-chain in the active site.[241] The carboxylic acid of the glycine occupies the 2OG binding pocket, forming interactions with the basic residue that binds the 2OG C-5 carboxylate (which is an arginine in the case of the PHDs). These generally rather flat heterocycles are predicted to fit snugly into the slot-shaped opening of the PHD active site, as evidenced by the crystal structures of two such compounds in complex with PHD2 (Fig. 13).

Alternative strategies which eschew the glycinamide motif have also been pursued (Fig. 14). Such compounds are also iron chelators and include functionalised 8-hydroxyquinolines,[75,242] pyrazolopyridines,[243] spiro[indoline-3,4′-piperidine]-2-ones,[244] and pyridinyldihydropyrazolones.[245] Finally, carboxymethyl-*N*-hydroxythiazoles are also potent PHD2 inhibitors, potentially chelating iron through the *N*-hydroxy and imino moieties.[246]

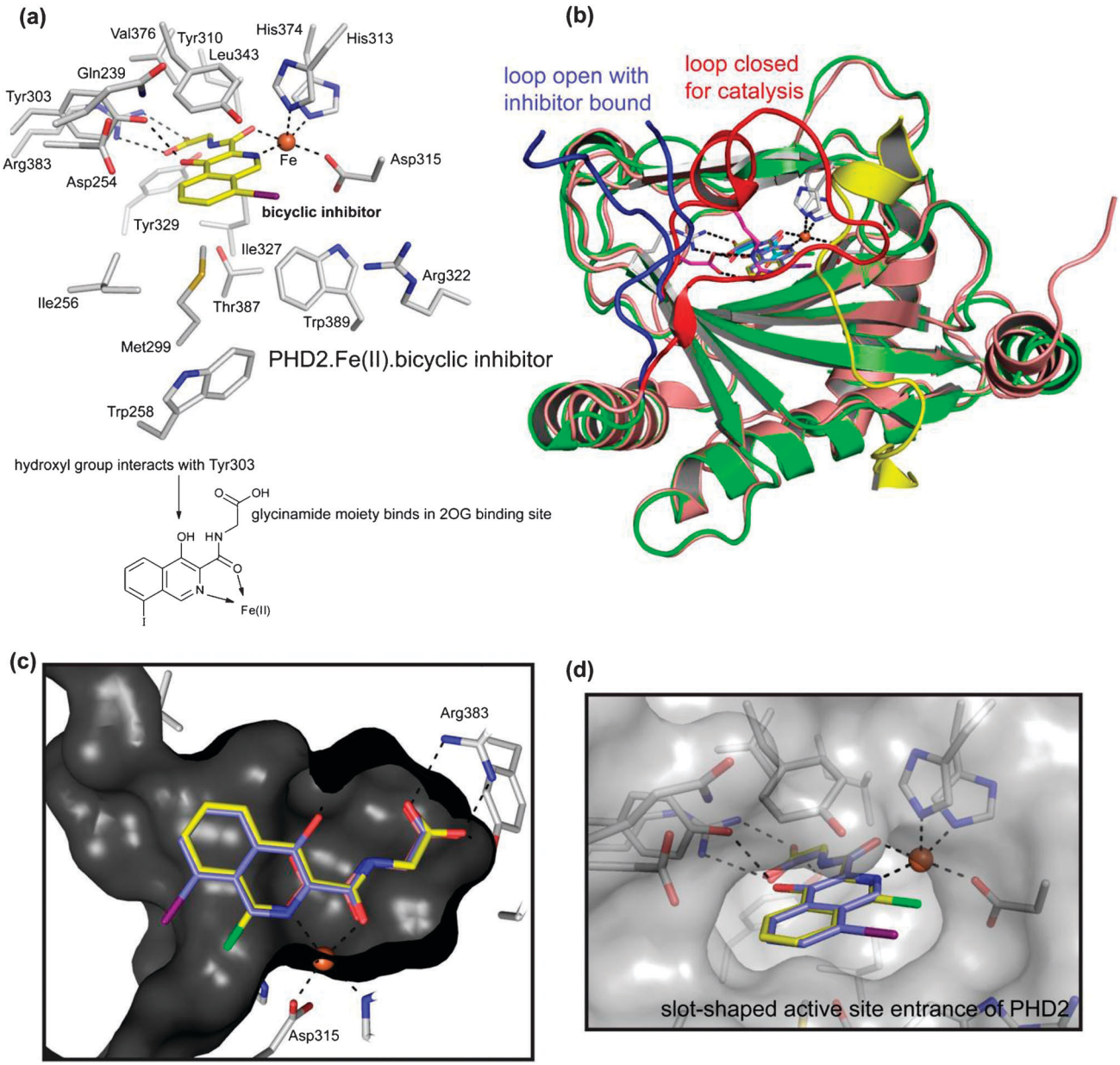

Fig. 13 Structures of the HIF prolyl hydroxylases complexed with inhibitors. (a) View from the crystal structure of PHD2 in complex with the bicyclic glycinamide 3-hydroxy-7-iodo-isoquinolinyl-2-(*N*-glycinyl)-carboxamide (PDB ID 2G19). (b) Views from crystal structures of PHD2 in complex with (i) a fragment of the HIF1-α substrate and NOG (substrate in yellow, protein shown in pink), and (ii) with bicyclic inhibitor (with protein shown in green). A mobile loop (shown in red) folds to enclose the substrate peptide for active catalysis to occur. When the inhibitor is bound, the inhibitor prevents the closure of the loop by disrupting an intra-loop salt bridge (the loop in blue in this structure). (c) The glycinamide moiety (of both FG-2216 and the 7-iodo bicyclic inhibitor) binds in the 2OG binding site, forming a salt bridge with Arg383, while the 3-hydroxyl group hydrogen bonds with Tyr303. (d) The entrance to the active site of PHD2 in the inhibitor structures is relatively flat and 'slot-shaped,' and most of the reported potent inhibitors are relatively flat aromatic compounds that can fit into this 'slot.'

10.6 Inhibition of the histone demethylases

Histone lysine demethylases remove methyl groups from N^{ε}-methyl lysine residues in the N-terminal tails of histone proteins (Fig. 15), which package DNA into chromatin. Post-translational modifications (including acetylation, methylation and phosphorylation, among others) to histone tails are involved in the regulation of transcription (Fig. 15), and many enzymes that place (acetyltransferases, methyltransferases and kinases) and remove (deacetylases, demethylases and phosphorylases) these modifications have been identified (for reviews, see ref. 247 and 248).

Since the initial report that FBXL11/KDM2A was a 2OG and iron dependent histone H3K36me2 demethylase (*i.e.* acting on the N^{ε}-dimethyllysine state of Lys36 of human histone H3),[66] demethylases that target H3K4, H3K9, H3K27, H3K36 and H4K20 residues have also been reported.[249] Uniquely, these enzymes act on all three possible methylation

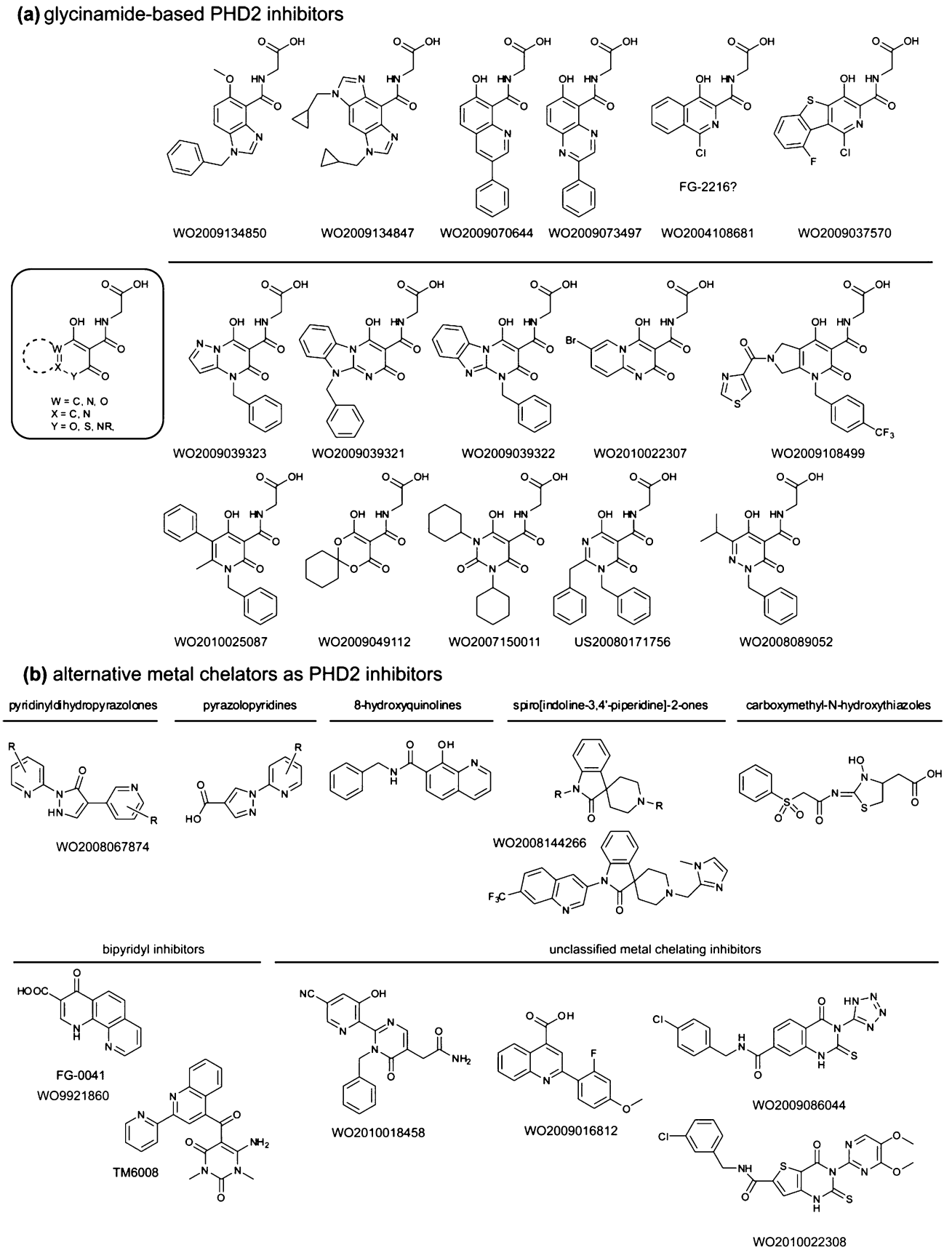

Fig. 14 Development of inhibitors of the HIF prolyl hydroxylases. (a) Variations on the glycinamide motif have been developed as inhibitors of PHD2. Selected compounds are shown; see ref. 238 for more detailed review. (b) Alternative metal chelation motifs have also been developed. Where applicable, selected patent applications associated with these compounds are given.

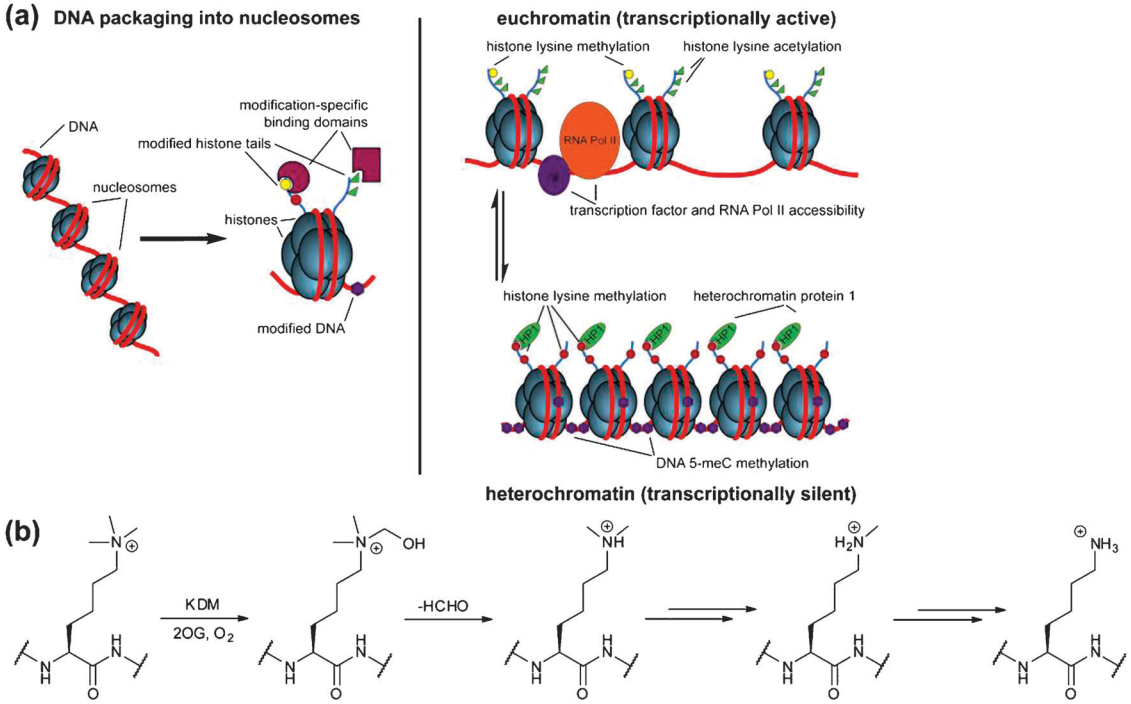

Fig. 15 Role of histone demethylases in chromatin modification. (a) Eukaryotic DNA is packaged into chromatin by formation of nucleosomes, which consist of eight histone proteins around which is wrapped ~146bp of DNA. Post-translational modifications of amino acid residues in the histone tails contribute to regulation of transcription by establishing euchromatin (transcriptionally active/accessible to RNA polymerase II) or heterochromatin (transcriptionally silent) states.[247] Note the figure is a simplification, many proteins are involved in transcriptional regulation. (b) The JmjC subfamily of 2OG oxygenases catalyse N^{ε}-demethylation of all three N^{ε}-methylated forms of histone lysine residues.

states of N^{ε}-methyl lysine residues. The biological roles of individual demethylases are still being deciphered, and the contributions of particular demethylases to disease have still to be fully elucidated (reviewed in ref. 25 and 248). Several examples given here will serve to show that while some histone demethylases may be attractive potential therapeutic targets for the treatment of cancers, others are possible tumour suppressors, suggesting that their roles in normal and cancer biology may be complex. Other potential therapeutic uses for KDM inhibitors include stem cell regulation, and the treatment of inflammatory and neurological disorders.

The available evidence is that structurally similar histone demethylase enzymes/domains of larger proteins can have very different and even opposing biological roles. For example, there is evidence that FBXL10, a K36me2 demethylase, is a tumour suppressor gene, acting as a transcriptional repressor of *c-Jun* target genes; inhibition of its expression causes increased cell proliferation.[250] In contrast, the catalytic domain of PHF8, which is structurally related to FBXL10,[251] has been shown to be essential for normal embryonic development, with mutations in the *PHF8* gene leading to midline defects.[252–255] Another example of apparently similar enzymes with different biological roles comes from the JMJD1 subfamily. JMJD1B, a K9me2 demethylase, is reported to have possible tumour suppressor activity.[256] JMJD1A, also a K9me2 demethylase, is a coactivator of androgen-receptor mediated transcription, and is essential for spermatogenesis, with disruption of JMJD1A activity causing male infertility.[257] From a therapeutic perspective, most interest on the 2OG histone demethylases has centred on possible links with cancer:

There is evidence that the JMJD2 demethylases are necessary for androgen receptor dependent transcription of certain genes, including prostate specific antigen; consistent with this role, JMJD2A-C are upregulated in prostate cancers.[258,259] The JMJD2 demethylases are also upregulated in oesophageal squamous cell carcinoma cells, where inhibition of their expression by siRNA has been shown to decrease proliferation.[259] JARID1B prevents terminal differentiation in embryonic stem cells, and is proposed to contribute to proliferation by repressing negative regulators of cell growth in both prostate and breast cancers, where it is overexpressed.[260,261] JARID1A is likewise overexpressed in gastric cancer cells, where its inhibition by RNAi leads to senescence.[262] JARID1C, however, is expressed primarily in neuronal tissues, and mutations reducing catalytic activity are associated with X-linked mental retardation;[263] JARID1C mutations have also been identified in renal carcinomas.[264] The JMJD3/UTX demethylase family has several identified biological roles, including activation of genes involved in developmental morphogenesis, stem cell self-renewal and cellular responses to inflammation.[265] JMJD3 has been shown to be upregulated in metastatic prostate cancers, but has also been described as a tumour suppressor gene,[265–267] and UTX is mutated in renal carcinomas.[264]

The above examples demonstrate that histone demethylases with the same biochemical catalytic function (*i.e.* demethylation of the same histone lysine residue) may have dramatically

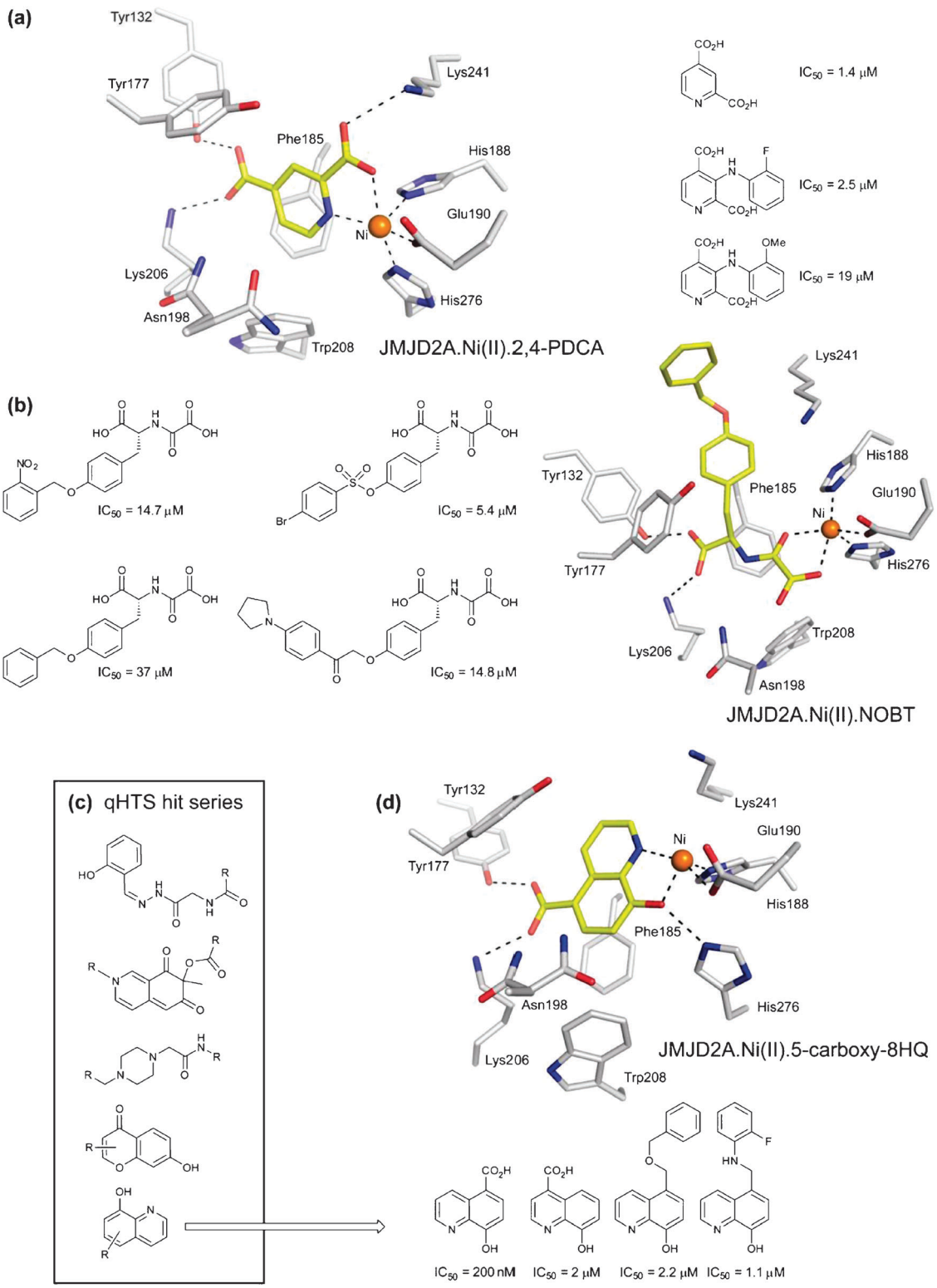

Fig. 16 Inhibitors of the JMJD2 histone demethylases. (a) View from a crystal structure of JMJD2A in complex with 2,4-PDCA (PDB ID 2VD7). The crystal structure indicates that substituents at the 3-position of 2,4-PDCA would be tolerated by the enzyme; 3-arylamino derivatives of 2,4-PDCA were shown to inhibit JMJD2 enzymes potently, but do not inhibit PHD2. (b) View from the crystal structure of JMJD2A in complex with *N*-oxalyl-D-(*O*-benzyl)tyrosine. Several other variations on the *N*-oxalyl-D-tyrosine scaffold are also good inhibitors of the JMJD2 demethylases, with sulfonate derivatives among the most potent. These inhibitors bind in the 2OG binding site but also block peptide substrate binding. (c) Selected compound classes identified as hit series from a high-throughput screen against the demethylase JMJD2E. (d) View from a crystal structure of JMJD2A in complex with the inhibitor 5-carboxy-8-hydroxyquinoline, identified through high-throughput screening, together with structures of several other potent inhibitors of the JMJD2 demethylases based on the 8-hydroxyquinoline template.

different roles in cancer or other diseases. This is perhaps unsurprising, given that many 'non-catalytic' factors likely affect the means by which histone demethylases contribute to transcriptional regulation. Thus, while some of the histone demethylases may have roles in cancer or other diseases which might be suitable for therapeutic intervention by use of small molecule inhibitors of these enzymes, the available data are limited, and the confirmed roles of many histone demethylases in normal development suggests that the 'rational' therapeutic inhibition of these enzymes may be challenging, given their roles in general transcriptional regulation.

There have been several reported efforts to develop inhibitors for the 2OG dependent histone demethylases, both as chemical probes to elucidate function, and as potential leads for therapeutic applications. A variety of assay methods have been applied for these enzymes, including formaldehyde detection[71,268] and immuno-detection of demethylated substrate peptides.[73] To date, two high-throughput screens for inhibitors of demethylases from the JMJD2/KDM4 subfamily have been reported (PubChem assay IDs 2421 (JMJD2E) and 2123 (JMJD2C));[70,71] and these represent the first publically available high-throughput screens against any of the 2OG oxygenases. To date, three classes of compound have been the focus of efforts to develop inhibitors of the 2OG dependent histone demethylases (Fig. 16).

N-Oxalyl amino acids were some of the first compounds demonstrated to be histone demethylase inhibitors (Fig. 16).[68,269] Development of the *N*-oxalyl amino template, employing structural and dynamic techniques including protein mass spectrometry, was used to identify more potent and selective inhibitors. It was found that *N*-oxalyl-D-amino acid derivatives showed selectivity of inhibition for the JMJD2 histone demethylases over the HIF prolyl hydroxylase PHD2.[95]

Hydroxamic acids also inhibit 2OG dependent histone demethylases, with the histone deacetylase inhibitor Vorinostat/SAHA shown to inhibit the JMJD2 demethylases with micromolar potency.[68] Hydroxamic acid derivatives of succinate were also identified as histone demethylase inhibitors, with reported selectivity for the JMJD2 demethylases over PHD1/PHD2.[101] These results raise the possibility that some of the biological effects of the hydroxamic acid HDAC inhibitors may be due to 2OG oxygenase inhibition.

A high-throughput screen for inhibitors of the demethylase JMJD2E identified several classes of demethylase inhibitors, including 8-hydroxyquinoline derivatives, which were demonstrated to inhibit *in vitro* with nanomolar potency, as well as inhibiting demethylases in cells (Fig. 16).[70] Although hydroxyquinolines have also been described as inhibitors of the HIF PHDs[75,242] and FIH,[270,271] the structural analyses indicate that modifications to this template will enable selective inhibition of the histone demethylases. Crystal structures have been reported for 8-hydroxyquinolines in complex with both FIH and JMJD2A, which should enable future design of selective inhibitors for these enzymes.[70,271]

A novel approach to inhibition of the JMJD2 demethylases has employed compounds that eject Zn(II) ions from Cys/His Zn-binding sites (Fig. 17). These compounds, which included disulfiram and ebselen, ejected a structural Zn(II) ion from the catalytic domain of the JMJD2 enzymes, leading to irreversible catalytic inactivation of the enzyme *in vitro*.[27] This approach may be useful for achieving selective inhibition of particular oxygenase subfamilies, as many 2OG oxygenases do not contain structural Zn(II) binding sites, though achieving selectivity over other Zn(II) binding proteins is likely to be challenging.

11. Conclusions

The past decade has seen significant advances in our understanding of the functions of human 2OG oxygenases. The discovery of roles for the HIF hydroxylases in hypoxic sensing has also stimulated widespread interest in their inhibition for therapeutic applications in anaemia and ischaemic disease. The results of clinical trials of the first wave of such

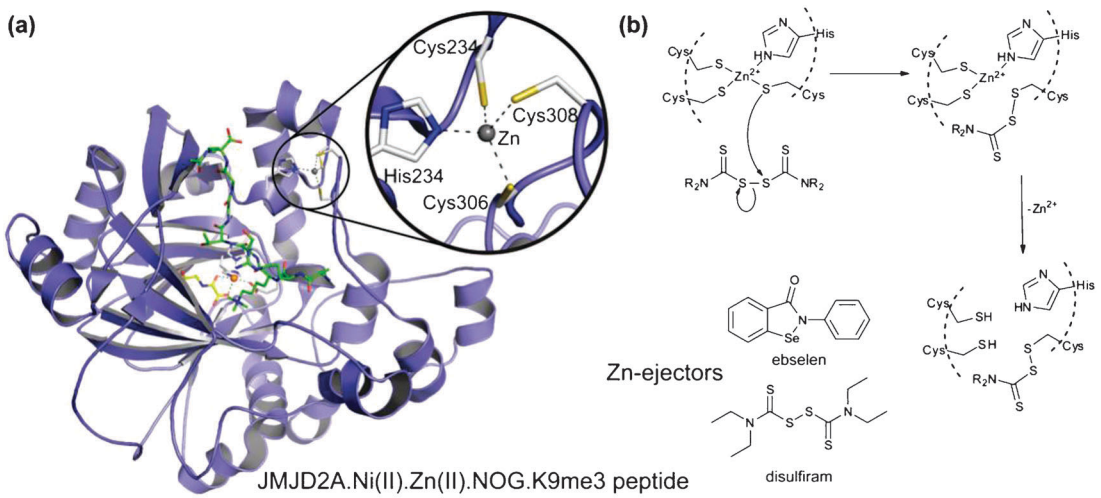

Fig. 17 Inhibition of the histone demethylase JMJD2A by ejection of structural Zn. (a) View from a crystal structure of JMJD2A in complex with H3K9me3 substrate peptide (PDB ID 2OQ6). Zn(II) is chelated by three cysteines and one histidine residue, and serves to hold together two loops of the enzyme. (b) The binding of Zn(II) is disrupted by 'Zn-ejectors' such as disulfiram and ebselen; a possible mechanism is shown here.

compounds should soon be available and will give an indication of how readily these enzymes can be targeted.

The roles of 2OG oxygenases in fatty acid metabolism have already been exploited in the inhibition of carnitine biosynthesis, and there is further potential in this field. In particular, the protein product of *FTO*, a gene first identified in genome wide association studies as being associated with obesity, has been shown to be a nucleic acid *N*-demethylase.[81,82] It is not yet known how nucleic acid *N*-methylation states regulate body mass (if indeed there is a direct connection). However, several model studies suggest that FTO inhibition is likely to decrease body mass.[272–274]

The role of 2OG oxygenases in epigenetic regulation has attracted significant attention. To date, most focus has been on the histone modifying N^{ε}-methyl lysine demethylases, with exploratory pharmaceutical work in progress, primarily (to our knowledge) targeted for cancer, in part stimulated by the clinical success of histone deacetylase inhibitors. However, the complexity of the role of methylation in the regulation of gene expression and the epigenetic transfer of information (*i.e.* between generations, without changes in the 'ATCG' DNA sequence) means that the rational targeting of specific histone modifying enzymes is at an early stage, and it is difficult to predict the effects of small molecule inhibitors in animals. This is also true for the 2OG oxygenases involved in DNA and RNA modifications. However, the discovery that the TET enzymes play widespread roles in hydroxymethylcytosine biosynthesis is an exciting one from a basic science perspective,[80,275,276] and raises the possibility that gene expression may be regulated by oxygen in a more direct manner than that mediated by the HIF hydroxylases. We are particularly interested in defining the roles of other 2OG oxygenases that are involved in the oxygen dependent regulation of gene expression. However, to date the HIF hydroxylases are the only family where this connection has been clearly identified and the mechanisms involved elucidated fully.

In terms of inhibition, work on the human 2OG oxygenases should be regarded as being at a relatively early stage, at least from a medicinal chemistry perspective. However, there is reason to propose that as a family they should be tractable targets. This is because there are relatively few 2OG oxygenases in humans, their active sites are amenable to small molecule inhibition, they have a range of substrates (compared to proteases/kinases) that should help to enable selective inhibition, and the results to date indicate that their inhibition is at least not highly toxic.

Our objective, together with others in the academic community, is comprehensively to define roles for 2OG oxygenases, at biochemical, cellular and physiological levels. We also aim to define the structures of all human 2OG oxygenases and to identify small molecules that selectively inhibit specific enzymes for use in functional assays. We hope that these efforts will help to enable the manipulation of the 2OG oxygenases with useful therapeutics.

Acknowledgements

We thank the Wellcome Trust, the Biological and Biotechnological Research Council, the European Union, and Pfizer for funding our research. We apologise for incomplete citations due to space constraints. We have only cited patent applications where specific compound structures are not in the academic literature. We thank our colleagues for discussion and support.

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