Institution: University of Oxford

Unit of Assessment: 8. Chemistry

Title of case study: UOA08-03: Oxygenases – from Chemistry to Medicine

1. Summary of the impact

Breakthrough structural and mechanistic work at Oxford University investigating how enzymes catalyse oxidising reactions has had major impacts in biomedicinal fields, including how humans adapt to changes in oxygen availability. Impacts arising from the work since 2008 include the identification of new drug targets for major diseases ranging from anaemia to cancer that are being clinically pursued by pharmaceutical companies (including GSK, Bayer, Astellas, Akebia) and smaller companies (including the Oxford spin out ReOx), and the sale of products including small-molecule probes (e.g. by Tocris, Millipore, Selleck Chem) that are of use in biomedicinal/pharmaceutical research, especially in the emerging field of epigenetics.

2. Underpinning research

Work from the Schofield group and collaborators at Oxford University on oxygenases - enzymes that can catalyse transformations not presently possible using synthetic methodology - has led to structural and mechanistic insights of widespread academic, medicinal, and commercial interest. Studies on bacterial enzymes in antibiotic biosynthesis resulted in pioneering structure determinations including of substrate complexes for oxygenases, e.g. [1]. With a desire to apply their skills to human biology and disease the group focused its activities (1996 onwards) to address mechanisms, structures and functions of the ~60 human 2-oxoglutarate (2OG) oxygenses with assigned and unassigned roles (at the time the majority were unassigned). Initial work on oxygenases involved in chlorophyll metabolism resulted in pathophysiological insights by correlating chemical and clinical data. This work placed the group in an excellent position to address functions of unassigned 2OG oxygenases in an interdisciplinary approach employing synthesis, biochemistry, structural and cell biology, methods of genetic and chemical intervention, structure-informed bioinformatics, and clinical data. The results have had major impact by identifying new signalling mechanisms and therapeutic targets. Studies on metabolic oxygenases included assignment of the fat-mass and obesity-associated protein FTO (mutations of which correlate with obesity) as a demethylase [2], raising the possibility that metabolism is regulated by nucleic acid methylation, a finding that has stimulated research worldwide.

Working with Peter Ratcliffe (Dept of Medicine, Oxford University), a joint programme from 2000 onwards was initiated to elucidate molecular mechanisms by which animals respond to hypoxia, a long-standing physiological problem. Arising in this programme, the finding that oxygenases act as oxygen sensors [3 - 5] was a landmark discovery (>> 10000 citations for related papers). Following the discovery that the hypoxia inducible transcription factors (HIF) are O_2 regulated (by Semenza in the U.S.), an objective was to identify mechanisms of O_2-dependent HIF-degradation. Prolyl-hydroxylation was shown to regulate HIF levels, via increasing its binding to the von Hippel-Lindau protein, a ubiquitin ligase component. Structural knowledge enabled identification of candidates encoding for HIF prolyl-hydroxylases, leading to identification of 3 human HIF hydroxylase enzymes, PHD1-3, which act as hypoxia sensors [3]. When O_2 is limiting, HIF-hydroxylation slows, causing its concentration to rise, so triggering the hypoxic response. Crucially, work of the Oxford University team in 2000 demonstrated that small-molecules (developed by Oxford Chemistry) mediate HIF prolyl-hydroxylase inhibition, leading to upregulation of HIF target genes, including those of major therapeutic benefit such as erythropoietin (EPO).

Following the finding that O_2-dependent HIF Asn hydroxylation reduces its transcriptional activity, Oxford work revealed that factor-inhibiting HIF (FIH), a JmjC protein, is the HIF Asn-hydroxylase [4]. Contrary to the paradigm, subsequent work demonstrated that hydroxylation of intracellularly localised proteins is common, opening a new protein-research field. Sequence analyses, informed by structures determined in Oxford, revealed that many genes encode for oxygenases involved in chromatin regulation. Following the discovery that many JmjC enzymes are histone demethylases, a programme to investigate oxygenase roles in epigenetics was initiated. Highlights include

structures for demethylases complexed with substrates, discovery of splicing factor hydroxylases, identification of a demethylase associated with X-linked mental retardation / cleft lip palate diseases [6], and of the ribosomal oxygenases. The collective results suggest all steps in protein expression are regulated by oxygen availability, and have opened up new therapeutic targets.

Key Oxford University contributors: C.J. Schofield, S. J. Conway, B. G. Davis, E. Flashman and A. Kawamura groups in Chemistry Department; P.J. Ratcliffe and C.W. Pugh in Medicine Department (work on hypoxia); C. Bountra and colleagues in the Structural Genomics Consortium (work on epigenetic probes). Schofield and Ratcliffe/Pugh collaborated from 2000 onwards; Schofield and SGC from 2005 on probe research.

3. References to the research

Asterisked outputs denote best indicators of quality;

1. Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase, Zhang ZH, Ren JS, Stammers DK, Baldwin JE, Harlos K, Schofield CJ, Nature Structural Biology 7: 127-133, 2000. DOI: 10.1038/72398. *Pioneering structural studies of a 2-oxoglutarate enzyme-substrate complex*.

2. The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase, Gerken T, et al., Science 318: 1469-1472, 2007. DOI: 10.1126/science.1151710.

3. *C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation, Epstein ACR, et al., Cell 107: 43-54, 2001. DOI: 10.1016/S0092-8674(01)00507-4. *Identification of the HIF prolyl-hydroxylases – the work enabled the prosecution of new therapeutic targets by small-molecule pharmaceuticals*.

4. *Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family, Hewitson KS, et al, J. Biol. Chem. 277: 26351-26355, 2002. DOI: 10.1074/jbc.C200273200. *Identification of a FIH, as the HIF Asn-hydroxylase, and consequently of the family of JmjC proteins/enzymes as oxygenases, opening up a new field in transcriptional regulation*.

5. *Oxygen sensing by HIF hydroxylases, Schofield CJ and Ratcliffe PJ, Nat. Rev. Mol. Cell Biol. 5: 343-354, 2004. DOI: 10.1038/nrm1366. *Exemplary studies on the role of oxygenases in the regulation of gene expression*.

6. Jmjd6 Catalyses Lysyl-Hydroxylation of U2AF65, a Protein Associated with RNA Splicing, Webby CJ, et al., Science 325: 90-93, 2009. DOI: 10.1126/science.1175865.

Five Patent families relating to Oxford work on oxygenases have been filed by ISIS Innovation.

4. Details of the impact

The breakthrough discoveries on oxygenases have had specific impacts including the following.

Identification/validation of new drug targets for diseases ranging from anaemia to cancer that are being pursued by multiple companies [7 - 10].

Enabling work in the Oxford University laboratories (1996-2002) followed by that in the spin-out ReOx (2002 onwards), demonstrated that small-molecule mediated inhibition of the HIF prolyl-hydroxylases upregulates proteins of major medicinal relevance (e.g. EPO and vascular endothelial growth factor, VEGF), thus validating the HIF hydroxylases as drug targets (see Section 2). Subsequently, pharmaceutical companies, including GSK, Merck, Bayer, Fibrogen/Astellas, Akebia, and Johnson and Johnson have targeted the prolyl hydroxylase domain enzymes (PHDs), demonstrating very widespread interest in them as targets for the treatment of anaemia, and ischaemia-related diseases including heart disease and diabetes. At least 2 of these companies have PHD inhibitors which were progressed during 2008-2013 such that they are now in late-stage clinical trials, including for anaemia (worldwide market > £ 50 billion). For example, FibroGen, Inc. and Astellas Pharma Inc. have reported the initiation of clinical studies in the Phase 3 clinical development program of FG-4592/ASP1517, an orally administered small molecule

inhibitor of the HIF prolyl-hydroxylases, for treatment of anaemia associated with chronic kidney disease in patients, to support approval in the U.S. and Europe [7]. Phase 2 clinical studies showed that FG-4592/ASP1517 demonstrated "anaemia correction in treatment-naïve CKD patients not on dialysis as well as maintenance of hemoglobin in CKD patients on dialysis and not on dialysis." GSK have reported a "Phase IIa, Randomised, Single-Blind, Placebo-Controlled, Parallel-Group clinical study to Evaluate the Safety, Pharmacokinetics, and Efficacy of 28-day Repeat Oral Doses of GSK1278863A, a HIF prolyl-hydroxylase inhibitor, in anemic pre-dialysis and hemodialysis-dependent patients." [8]. At Bayer, "a Phase IIb program with the investigational new drug Molidustat (BAY 85-3934) is under initiation in patients with anaemia associated with chronic kidney disease and/or end-stage renal disease. Molidustat is a novel inhibitor of hypoxia-inducible factor (HIF) prolyl hydroxylase (PH) which stimulates erythropoietin (EPO) production and the formation of red blood cells. Phase I data have shown that inhibition of HIF-PH by Molidustat results in an increase in endogenous production of EPO." [9] Akebia report that "AKB-6548 and AKB-4924 are 2 selective HIF-PH [HIF prolyl-hydroxylase] inhibitors" ….."have profound effects for anaemia, wound healing and anti-microbial therapy. Akebia's HPTPβ inhibitors modulate Angiopoietin-2 activity and represent an exciting new approach in the treatments for vascular leak, retinopathy, cancer and critical limb ischemia (CLI)" [10]

Oxford's identification of one of the HIF hydroxylases as a JmjC protein stimulated work leading to their assignment as histone/chromatin demethylases acting on all 3 *N*-epsilon methylation states of lysine. Because of the fundamental role of histone methylation in the regulation of gene expression, sometimes in an epigenetic manner, the JmjC histone demethylases have also attracted considerable attention as potential targets for diseases ranging from cancer to genetic diseases (by several companies including GSK, Genentech, and Epitherapeutics). Andrea Cochran of Genentech states [11] "Oxford is one of a very small number of academic institutions at the leading edge of early-stage drug discovery and target validation in epigenetics" and "Each of the specific examples cited [relating to oxygenases and bromodomains] addresses problems that I have heard discussed internally multiple times and that have real impact on our ability to develop drugs against these targets."

Commercial sale of products including (i) small-molecules arising directly from Oxford work, (ii) antibodies arising directly and indirectly from Oxford work [12-13].

Oxford Chemistry work and that of its collaborators has directly or indirectly resulted in commercially available products for biomedical research. With respect to 2OG oxygenase/demethylase inhibitors, the Schofield group initiated a joint Wellcome Trust-funded project with the Structural Genomics Consortium, the NIH and (ultimately) more than 10 industrial partners and the National Institutes of Health, USA, to identify and distribute small molecule inhibitors of 'epigenetic' enzymes/proteins for use in probing biological function. Duncan Crawford, CSO of Tocris Bioscience, says [13] "This research has helped to open up an entirely new product line for (Tocris) in the past 3 or 4 years by generating small molecule probes for epigenetic research. We think this is likely to remain a very exciting field for us for the next decade or longer."

Some of the oxygenase/demethylase inhibitors arising from work in the Oxford Chemistry laboratories (in most cases in collaboration) are now commercially available, for example:

- Tocris Biosciences sells the following small molecules relating to Oxford precompetitive work on oxygenases and epigenetics: Daminozide, IOX1, IOX2, JQ1, DMOG, GSK J1, GSK J4 (arising from collaboration with GSK), OXFBD 02 and OXFBD 03.
- Cayman Chemical Company sells the following small molecules: Daminozide, IOX1, IOX2, JQ1 and DMOG.
- Selleck Chemicals sells the following small molecules: IOX2, JQ1, GSK J1 and GSK J4.
- SIGMA-ALDRICH sells the following small molecules: Daminozide, IOX1, IOX2, DMOG, GSK-J1 and GSK-J4.
- Millipore sells the following small molecules: Daminozide, IOX1, DMOG, GSK J1 and GSK J4.
- BioVision Inc. sells the following small molecules: IOX1, DMOG, GSK J1 and GSK J4.
- Axon Medchem sells the following small molecules: IOX2, GSK J1 and GSK J4.

- Bertin Pharma sells IOX1.
- Enzo Life Sciences sells DMOG.
- Many companies sell antibodies based on our discoveries relating to the hydroxylation of HIF: Hydroxy-HIF-1α (Pro564) (D43B5) XP® Rabbit mAb #3434 through Cell Signaling; Anti-HIF1 alpha (Hydroxy P402) antibody (ab72775) through Abcam; Anti-HIF-1-alpha Antibody, hydroxyproline (Pro402) through Millipore; HIF-1 alpha hydroxy P564 Antibody through Rockland.

Representative sales figures for probes (for oxygenases and bromodomains) based on Oxford University research from individual retailers (to September 2013): Tocris Biosciences [13] have distributed a total of 280 units (9 units of IOX1, 21 units of IOX2, 62 units of GSK J1, 121 units of GSK J4, and 59 units of JQ1), generating total revenue based on list prices in excess of £ 50000. Cayman Chemical Company has distributed 453 units of JQ1, 17 units of IOX1, and 26 units of IOX2, generating a total revenue in excess of £25000. Millipore has distributed 21 units of IOX1, generating a total revenue based on list prices of £2499. Note IOX1, IOX2, GSK J1, are oxygenase inhibitors; JQ1 is bromodomain inhibitor; further (>5) oxygenase and bromodomain inhibitors will be available in 2014. The geographical spread of sales includes 16 countries in North America, Australia, the Far East and Europe, with the purchases being approximately equally divided between academia and industry/government.

5. Sources to corroborate the impact (indicative maximum of 10 references)

[7] Clinical trials by Fibrogen /Astellas using HIF prolyl hydroxylases as targets: http://www.businesswire.com/news/home/20121211006761/en/FibroGen-Astellas-Announce-Initiation-Phase-3-Trial and Phase 2 trials at Fibrogen: http://www.fibrogen.com/press/release/pr_1352127487.

[8] GSK clinical trials using HIF prolyl hydroxylases as targets: http://www.gsk-clinicalstudyregister.com/protocol_detail.jsp;jsessionid=8CD9309F891F132231AB97DBE2D4AC09?protocolId=112844&studyId=81B449A5-23FF-4617-BAAE-FC6065229339&compound=GSK1278863.

[9]:Phase 1 trials at Bayer Pharma: http://www.bayerpharma.com/en/research-and-development/development-pipeline/index.php?phase=1 (http://www.investor.bayer.com/news/investor-news/investor-news/showNewsItem/1627/1381211700/d884bbd065/).

[10] Use of target by Akebia: http://www.akebia.com/research.html.
For a summary of industrial work on HIF hydroxylase inhibitors, see: Expert Opin. Ther. Pat. 2010, 20, 219-45. doi: 10.1517/13543776.2010.510836 .

[11] Letter from Genentech Principal Scientist (Nov 3rd, 2013) concerning Oxford's work on demethylases/oxygenases and bromodomains (held on file).

[12] Tocris Biosciences website, http://www.tocris.com/ under hydroxylase and demethylase categories; Cayman Chemical Company, http://www.caymanchem.com; Selleck Chemicals, www.selleckchem.com; SIGMA-ALDRICH, http://www.sigmaaldrich.com; Millipore, http://www.millipore.com/; BioVision Inc., http://www.biovision.com; Axon Medchem, http://axonmedchem.com; Bertin Pharma, http://bioreagent.bertinpharma.com/product-18511.aspx; Enzo Life Sciences, http://www.enzolifesciences.com/BML-EI347/dimethyloxaloylglycine/

[13] Letter from Tocris Bioscience CSO (Oct 4th, 2013) concerning Oxford's work on target validation and inhibitors (held on file).