DESIGN, SYNTHESIS, *IN SILICO* AND *IN VITRO* EVALUATIONS OF NEW INHIBITORS TARGETING HUMAN HYPOXIA INDUCIBLE FACTOR (HIF) PROLYL HYDROXYLASE DOMAIN (PHD) ENZYMES

SAYAF ABRAR MOHAMMAD

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by

SAYAF ABRAR MOHAMMAD

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2OG	2-oxoglutarate
2OGXs	2-oxoglutarate-dependent dioxygenases
ADMET	Absorption, distribution, metabolism, excretion, and toxicity
ANPDB	African Natural Products Database
AMES	Ames test for mutagenicity
ALK	Anaplastic lymphoma kinase
ACE	Angiotensin-converting enzyme
AI	Artificial intelligence
ARNT	Aryl hydrocarbon receptor nuclear translocator
AutoQSAR	Automated quantitative structure-activity relationship
BBB	Blood-brain barrier
CDs	Carbamoyl derivatives
AMS	Carbamoyl derivatives (AMS-1 to AMS-19)
CABPIs	Carboxylic acid-based PHD inhibitors
CVDs	Cardiovascular diseases
CPMG	Carr-Purcell-Meiboom-Gill (NMR binding analysis)
CNS	Central nervous system
CKD	Chronic kidney disease
CPPTRAJ	Command-line processing and programming trajectory
CMNPD	Comprehensive Marine Natural Products Database
CADD	Computer-aided drug design
CAD	C-terminal transactivation domain
CYP450	Cytochrome P450

DL	Deep learning
DVT	Deep venous thrombosis
EANPDB	East African Natural Products Database
ESI-HRMS	Electrospray ionization high-resolution mass spectrometry
EPO	Erythropoietin
XP	Extra precision docking
FIH	Factor inhibiting HIF
FGFR	Fibroblast growth factor receptors
FDA	Food and Drug Administration
FT-IR	Fourier transform-infrared spectroscopy
FAF-Drugs4	Free ADME-Tox filtering software for drug discovery
GAFF	General AMBER force field
IC_{50}	Half maximal inhibitory concentration
HRMS	High-resolution mass spectrometry
HTS	High-throughput screening
HTVS	High-throughput virtual screening
HIA	Human intestinal absorption
HMG-CoA	Hydroxymethylglutaryl-CoA
HIF	Hypoxia inducible factor
IFD	Induced fit docking
ICAM-1	Intercellular adhesion molecule 1
JMJD6	Jumonji domain-containing protein 6
LBDD	Ligand-based drug design
RO5	Lipinski’s Rule of Five
LFA-1	Lymphocyte function-associated antigen-1

KDM4A	Lysine-specific demethylase 4A
ML	Machine learning
MD	Molecular dynamics
MM/GBSA	Molecular mechanics/generalized Born surface area
4HG	N-[(4-Hydroxy-8-iodoisoquinolin-3-yl) carbonyl] glycine
NPACT	Naturally occurring plant-based anti-cancer compound-activity-target
pIC_{50}	Negative logarithm of the half-maximal inhibitory concentration
NCABPIs	Non-carboxylic acid-based PHD inhibitors
NCPIs	Non-conventional PHD inhibitors
NSCLC	Non-small-cell lung cancer
NOG	N-oxalylglycine
NMR	Nuclear magnetic resonance spectroscopy
OPC	Optimal point charge
OCT	Organic cation transporter
ODD	Oxygen-dependent degradation domain
PAMPA	Parallel artificial membrane permeability assay
PME	Particle mesh Ewald
P-gp	P-glycoprotein
PK	Pharmacokinetics
pkCSM	Predicting small-molecule pharmacokinetic and toxicity properties using graph-based signature
PHD	Prolyl hydroxylase domain
PDB	Protein Data Bank
PDBQT	Protein Data Bank, with charges and torsions

PPIs	Protein-protein interactions
QSAR	Quantitative structure-activity relationship
Rg	Radius of gyration
RCSB	Research Collaboratory for Structural Bioinformatics
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
RMSV	Root mean square variation
ROS1	ROS proto-oncogene 1
SNP	Single nucleotide polymorphism
SPE-MS	Solid phase extraction mass spectrometry
SASA	Solvent-accessible surface area
SANCDB	South African Natural Compounds Database
SPMSM	Specific PHD2 hydroxylation assay
SAR	Structure-activity relationship
SBDD	Structure-based drug design
TPSA	Topological polar surface area
TIP3P	Transferable intermolecular potential with 3 points
2D-QSAR	Two-dimensional quantitative structure-activity relationship
UCSF	University of California, San Francisco
VEGF	Vascular endothelial growth factor
VDss	Volume of distribution at steady state
VHL	Von Hippel-Lindau
pVHL	Von Hippel-Lindau protein

LIST OF APPENDICES

REKABENTUK, SINTESIS, PENILAIAN *IN SILICO* DAN *IN VITRO* PERENCAT BAHARU YANG MENSASARKAN ENZIM PROLIL HIDROKSILASE (PHD) FAKTOR PENDORONG HIPOKSIA MANUSIA (HIF)

ABSTRAK

Enzim prolil hidroksilase (PHD) faktor pendorong hipoksia (HIF) memainkan peranan penting dalam pengesanan oksigen dan pengawalan, menjadikannya sasaran terapeutik utama bagi penyakit berkaitan hipoksia, termasuk penyakit kardiovaskular (CVD), penyakit buah pinggang kronik (CKD), dan anemia. Walau bagaimanapun, perencat PHD sedia ada mempunyai kelemahan yang ketara, termasuk ketoksikan, risiko tromboembolik, dan kekurangan selektiviti, yang memerlukan penemuan alternatif yang lebih selamat dan berkesan. Kajian ini menggunakan pendekatan gabungan komputasi-eksperimen untuk mengenal pasti perencat PHD yang baharu dengan mengintegrasikan dok molekul, simulasi dinamik molekul (MD), pengiraan tenaga bebas, pemodelan QSAR, dan pengesahan eksperimen. Satu set data yang terdiri daripada 147,583 sebatian semula jadi dan sintetik daripada pelbagai pangkalan data (AfroDb, CMNPD, NANPDB, NEANPDB, EANPDB, SANCDB, pangkalan data REAL EnamineStore, dan repositori berasaskan ZINC) telah ditapis menggunakan teknik *in silico* yang maju. Saringan maya (VS) yang diikuti dengan dok keserasian-teraruh (IFD) telah mengenal pasti beberapa calon berpotensi, termasuk ZINC36378940, ZINC02104411, CMNPD13808, dan Z6742136823, yang menunjukkan pertalian pengikatan yang unggul (-13.34 hingga -8.69 kcal/mol) serta interaksi stabil dengan residu tapak aktif yang kritikal (Tyr303, Arg383, Tyr329, Trp389). Calon terbaik daripada IFD kemudiannya dinilai lebih lanjut melalui simulasi

MD, yang mengesahkan kestabilan pengikatan mereka. Eksplorasi *in silico* dan *in vitro* terhadap sebatian daripada pangkalan data REAL EnamineStore telah mengenalpasti tujuh sebatian aktif, dengan Z6742136823 muncul sebagai perencat PHD2 yang menjanjikan (IC_{50}: 5.27 μM). Dalam kajian ini, pelbagai rangka kimia yang berpotensi telah dikenal pasti, termasuk isoindol, pteridin, triterpenoid, spiro-isoindol, biflavonoid, kromon, β-laktam, indol, kuinolin, kromenon dan isoquinolin. Rangka-rangka ini menyediakan templat yang bernilai untuk reka bentuk masa hadapan perencat PHD. Selain itu, satu siri baharu terbitan karbamoil (CD) (**AMS-1** hingga **AMS-19**) telah didok, disintesis, dan dicirikan menggunakan FT-IR, NMR, dan HRMS. Meskipun keputusan dok *in silico* kelihatan menjanjikan, ujian enzim *in vitro* awal menunjukkan bahawa kebanyakan CD menunjukkan aktiviti perencatan yang lemah terhadap PHD2 dan PHD3, kecuali **AMS-12** yang menunjukkan perencatan berpotensi (PHD2 IC_{50}= 22.91 μM; perencatan PHD3= 76.42% pada 20 μM). Pemprofilan kepekaan enzim selanjutnya mendedahkan bahawa **AMS-12** mempunyai perencatan lemah terhadap FIH (IC_{50} = 116.4 μM) dan tiada aktiviti terhadap lisin demetilase 4A (KDM4A) atau protein yang mengandungi domain Jumonji 6 (JMJD6), terus menunjukkan kekhususannya. Analisis NMR Carr-Purcell-Meiboom-Gill (CPMG) menunjukkan bahawa **AMS-12** berkesan menyingkirkan ko-substrat 2-oksoglutarat (2OG) daripada PHD2, sekali gus mengesahkan potensi pengikatan kompetitifnya yang tinggi.

DESIGN, SYNTHESIS, *IN SILICO* AND *IN VITRO* EVALUATIONS OF NEW INHIBITORS TARGETING HUMAN HYPOXIA INDUCIBLE FACTOR (HIF) PROLYL HYDROXYLASE DOMAIN (PHD) ENZYMES

ABSTRACT

Hypoxia inducible factor (HIF) prolyl hydroxylase domain (PHD) enzymes play a pivotal role in oxygen sensing and regulation, making them key therapeutic targets for hypoxia-related diseases, including cardiovascular diseases (CVDs), chronic kidney disease (CKD), and anemia. However, current PHD inhibitors suffer from significant limitations, including toxicity, thromboembolic risks, and poor selectivity, necessitating the discovery of safer and more effective alternatives. This research utilises a computational-experimental methodology to discover new PHD inhibitors through the integration of molecular docking, molecular dynamics (MD) simulations, and free energy calculations, QSAR modeling, and experimental validation. A dataset of 147,583 natural and synthetic compounds from diverse databases (AfroDb, CMNPD, NANPDB, NEANPDB, EANPDB, SANCDB, the REAL database of EnamineStore, and ZINC-based repositories) was screened using advanced *in silico* techniques. Virtual screening (VS), followed by induced fit docking (IFD), identified several promising candidates, including ZINC36378940, ZINC02104411, CMNPD13808, and Z6742136823, which exhibited superior binding affinities (-13.34 to -8.69 kcal/mol) and stable interactions with critical active-site residues (Tyr303, Arg383, Tyr329, Trp389). The top hits from IFD were further evaluated through MD simulations, which confirmed their binding stability. *In silico* and *in vitro* exploration of compounds from the REAL database of EnamineStore further identified seven active compounds, with Z6742136823 emerging as a

promising PHD2 inhibitor (IC_{50} = 5.27 μM). Across these studies, multiple promising chemical scaffolds were discovered, including isoindole, pteridine, triterpenoid, spiro-isoindole, biflavonoid, chromone, β-lactam, indole, quinoline, chromenone and isoquinoline. These scaffolds provide valuable templates for the future design PHD inhibitors. Additionally, a novel series of carbamoyl derivatives (CDs) (**AMS-1** to **AMS-19**) was docked, synthesized, and characterized using FT-IR, NMR, and HRMS. Despite promising *in silico* docking results, preliminary *in vitro* enzyme assays showed that most CDs exhibited poor inhibitory activity against PHD2 and PHD3, except for **AMS-12**, which displayed potent inhibition (PHD2 IC_{50} = 22.91 μM; PHD3 inhibition = 76.42% at 20 μM). Enzyme selectivity profiling further revealed that **AMS-12** had weak FIH inhibition (IC_{50} = 116.4 μM) and no activity against lysine demethylase 4A (KDM4A) or Jumonji domain-containing protein 6 (JMJD6), confirming its specificity. Carr-Purcell-Meiboom-Gill (CPMG) NMR analysis demonstrated that **AMS-12** effectively displaced the 2-oxoglutarate (2OG) co-substrate from PHD2, confirming its strong competitive binding potency.

CHAPTER 1

INTRODUCTION

1.1 Background

Hypoxia, a condition resulting from reduced oxygen availability triggers several cellular responses facilitated by hypoxia inducible factors (HIFs). Transcription factors significantly influence hypoxia signaling through the regulation of genes related to metabolism, angiogenesis, and erythropoiesis (Lee et al., 2020). The regulation of HIFs is largely controlled by enzymes known as prolyl hydroxylase domains (PHDs), which act as oxygen sensors (Hirota et al., 2021). These enzymes also hydroxylate HIFs under normal oxygen (O_2) levels, marking them for degradation (Taylor et al., 2022). PHDs are present in multiple isoforms and are expressed across various tissues, allowing them to play a significant role in oxygen sensing and adaptation (Lee et al., 2020).

The function of PHDs relies on the cofactor 2-oxoglutarate (2OG), as well as iron and oxygen, which are essential for PHD enzymatic activity (Fiorini et al., 2024). Recently, therapeutic targeting of PHDs has gained significant attention, with computer-aided drug design (CADD) playing a key role in these efforts (Sahlgren et al., 2017). The two primary and advanced techniques in CADD are ligand-based drug design (LBDD) and structure-based drug design (SBDD). However, despite the critical applications of CADD in drug discovery, challenges remain particularly in accurately predicting the behaviour of complex biological systems (Bharatam et al., 2021).

1.2 Hypoxia

Hypoxia, derived from the Greek words *"hypo"* (under) and *"oxia"* (oxygen), refers to a condition where a specific region of the body or the entire body is deprived of an adequate oxygen supply (Guo et al., 2019). Hypoxia can affect the entire body, known as generalized hypoxia, or it can impact specific tissues, referred to as tissue hypoxia, potentially leading to severe health consequences. Hypoxia may arise from insufficient oxygen delivery to the lungs, perhaps caused by multiple reasons. This encompasses respiratory ailments such as chronic obstructive pulmonary disease (COPD) and pneumonia, elevated altitudes, and circulatory disorders that hinder oxygen transfer in the bloodstream (Zhou et al., 2022). Additionally, it may occur when oxygen consumption exceeds normal levels, such as during exercise, infection, or increased metabolic activity, leading to systemic or localized oxygen deprivation (Choudhury et al., 2018).

Researchers, healthcare professionals, and scientists have been captivated by the profound impact of hypoxia on cellular activities and its relationship with a variety of diseases. This condition can arise from multiple factors, including environmental circumstances, various health states, or altered physiological processes, which disrupt the delicate balance between oxygen supply and consumption (Hasegawa et al., 2018; Masoud & Li, 2015; Zhang et al., 2011).

The investigation of hypoxia dates to the 18th century, with the first reports detailing the effects of altitude on human performance and health. In the early 1900s, Christian Bohr and colleagues introduced the concept of the oxygen dissociation curve in their groundbreaking research. This framework laid the foundation for our understanding of how haemoglobin binds and releases oxygen in response to changes

in partial pressure (Semenza et al.,1992). In 1991, Semenza and his team identified a hypoxia inducible enhancer situated upstream of the human erythropoietin gene in the kidneys and livers of transgenic mice subjected to functional hypoxia induced by anaemia. (Semenza et al.,1992). The nuclear factor identified as responsible for the enhanced gene expression observed in these experiments is referred to as hypoxia inducible factor (HIF). In response to hypoxia, cells initiate a series of adaptive changes aimed at maintaining cellular homeostasis and ensuring survival (Miikkulainen et al., 2019). The hypoxic response is primarily mediated by the HIF transcription factor family, which governs genes associated with angiogenesis, oxygen transport, energy metabolism, and apoptosis (Yang et al., 2016). When oxygen levels are low, HIF triggers a transcriptional response that induces adaptive changes in cellular activities, modifies energy production processes, and enhances oxygen transport. However, chronic or severe exposure to hypoxia can contribute to the development of several diseases, including and cardiovascular diseases, neurological disorders and, cancer (Luo et al., 2022).

1.3 Hypoxia inducible factors (HIFs)

Hypoxia inducible factors (HIFs) are a small family of heterodimeric transcription factors consisting of three members: HIF-1, HIF-2, and HIF-3 (Foster et al., 2014). HIFs are primarily responsible for mediating the cellular effects of hypoxia. In 1992, Semenza and his postdoctoral researcher Wang demonstrated that these heterodimers comprise an oxygen-sensitive α-subunit and a stable β-subunit (Eales et al., 2016; Wang et al., 1993). Cellular responses to low oxygen levels are largely orchestrated by HIF, which activates genes involved in key processes such as metabolic reprogramming, and angiogenesis (Liberti et al.,2016). Additionally, HIF

regulates gene expression related to immunity, inflammation, stem cell maintenance, and tissue repair (Foster et al., 2014). HIF functions as a key mediator in various pathological and physiological processes, encompassing human development, wound healing, ischaemia, cancer, and chronic kidney disease (Braicu et al., 2019; Zappa et al., 2016).

1.4 The hypoxia signaling pathway

The activity of the HIF protein is predominantly governed by the transcriptional dynamics and stability of its α-subunits, namely HIF-1α, HIF-2α, and HIF-3α. These activities are controlled in an oxygen-dependent manner through post-translational modifications (Miao et al., 2022). HIF-1α exhibits widespread expression and is involved in various cellular processes, whereas HIF-2α demonstrates a more specialised tissue distribution and specific functions, including the regulation of erythropoietin (EPO) synthesis in the liver (Tanaka et al., 2016). In contrast, HIF-3α is less well understood, though it is known to negatively regulate the activities of HIF-1α and HIF-2α (Miao et al., 2022).

In normoxic conditions, two proline residues (Pro402 and Pro564 in human HIF-α subunits) in the oxygen-dependent degradation domains (ODDs) of HIF-α subunits undergo hydroxylation by PHD enzymes (PHD1-3). This hydroxylation designates HIF-α for ubiquitin-dependent degradation by the von Hippel-Lindau (VHL) E3 ligase complex. Figure 1.1 illustrates the structural of human HIF-1α, highlighting the basic helix-loop-helix (bHLH), *C*-terminal transactivation domain (CAD), Per-ARNT-SIM (PAS), *C*-terminal oxygen-dependent degradation domain (CODD), nicotinamide adenine dinucleotide (NAD), and *N*-terminal oxygen-dependent degradation domain (NODD) regions essential for its functional

interactions and regulation (Thévenod et al., 2022). Kaelin and his team were the pioneers in identifying that the VHL gene encodes the tumour suppressor protein pVHL, which is essential for regulating cellular responses to hypoxia (Hurst et al., 2016). Mutations in the VHL gene can prevent the degradation of HIF-α in normoxic conditions by bypassing the ubiquitination checkpoint, thus stabilizing HIF-α even when oxygen is present (Tirpe et al., 2019). Furthermore, an asparagine residue in the CAD of HIF-α (Asn803 in human HIF-1α) undergoes hydroxylation by the factor inhibiting HIF (FIH) enzyme. This alteration obstructs the interaction of HIF-α with the coactivator p300/CBP, hence reducing its transcriptional function (Fantis et al., 2023). In 2003, Tanimoto *et al.* demonstrated that a single nucleotide polymorphism (SNP) in the HIF1A gene (rs11549465) results in the substitution of proline with serine at position 582 (P582S). This polymorphism increases the transcriptional activity of HIF-1α by altering the properties of its binding sites and target genes (Javier et al., 2015; Javier et al., 2017; Fernández et al., 2017).

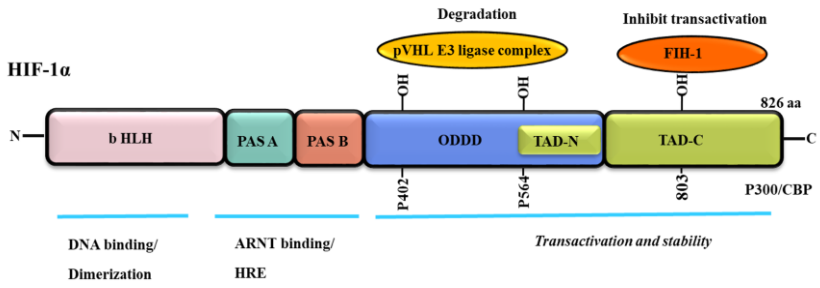

Figure 1.1 Structure of human HIF-1α: The schematic depicts key structural domains of HIF-1α involved in DNA binding, dimerization, ARNT interaction, and transcriptional regulation under hypoxic conditions. The basic helix-loop-helix (bHLH) and PAS (PER-ARNT-SIM) domains at the N-terminus mediates heterodimerization with HIF-β (ARNT) and recognition of hypoxia response elements (HREs) on target DNA. These domains are essential for DNA binding and the formation of a functional transcriptional complex. The N-terminal oxygen-dependent degradation domain (NODD) and C-terminal oxygen-dependent degradation domain (CODD) control protein stability through prolyl hydroxylation in normoxia. The N-terminal transactivation domain (NAD) and C-terminal transactivation domain (CAD) contribute to transactivation of hypoxia inducible genes and are also regulated by post-translational modifications and protein-protein interactions (Fernández et al., 2017).

The structural composition of human HIF-1α includes the bHLH and PAS domains, which are crucial for binding with HIF-β and the hypoxia response element (HRE). It comprises NODD, CODD, NAD, and CAD. The PAS domain is also associated with the period circadian protein (PER), aryl hydrocarbon receptor nuclear translocator (ARNT), and single-minded protein (SIM) (Javier et al., 2017; J Fernández et al., 2017).

PHDs and FIH are dioxygenases that require Fe (II) and 2OG for their enzymatic activity. These enzymes use oxygen and 2OG as co-substrates, producing succinate, carbon dioxide, and hydroxylated residues as byproducts. Figure 1.2 depicts hydroxylation reactions mediated by PHDs and FIH, highlighting the dependence on Fe (II), 2OG, and oxygen, with hydroxylated residues, succinate, and CO_2 as end products (Cavadas et al., 2013). The proposed mechanism of 2OG and Fe (II)-dependent oxygenases is illustrated in Figure 1.3. It outlines the process observed in various 2OG-dependent oxygenase reactions (Islam et al., 2018b; Martinez et al., 2015). Initially, 2OG interacts with Fe (II), displacing two water molecules within the enzyme's active site. Oxygen then reacts with 2OG, forming succinate and Fe (IV)=O via oxidative decarboxylation. The Fe (IV)=O hydroxylates the peptide substrate through a radical mechanism, altering its structure. Finally, the hydroxylated peptide and succinate exit, and the enzyme returns to its resting state (Tarhonskaya et al., 2014).

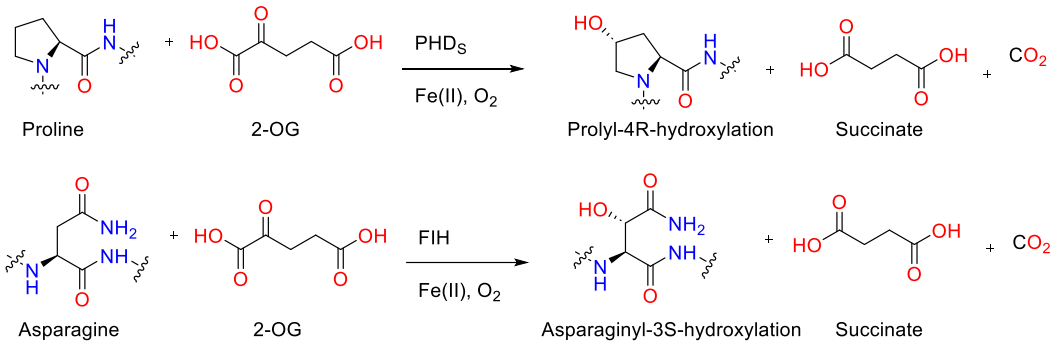

Figure 1.2 Hydroxylation of HIF-1α by PHDs and FIH, utilizing Fe (II), 2OG, and oxygen, results in proline/asparagine hydroxylation, with succinate and CO_2 as byproducts, crucial for regulating HIF stability and hypoxic response.

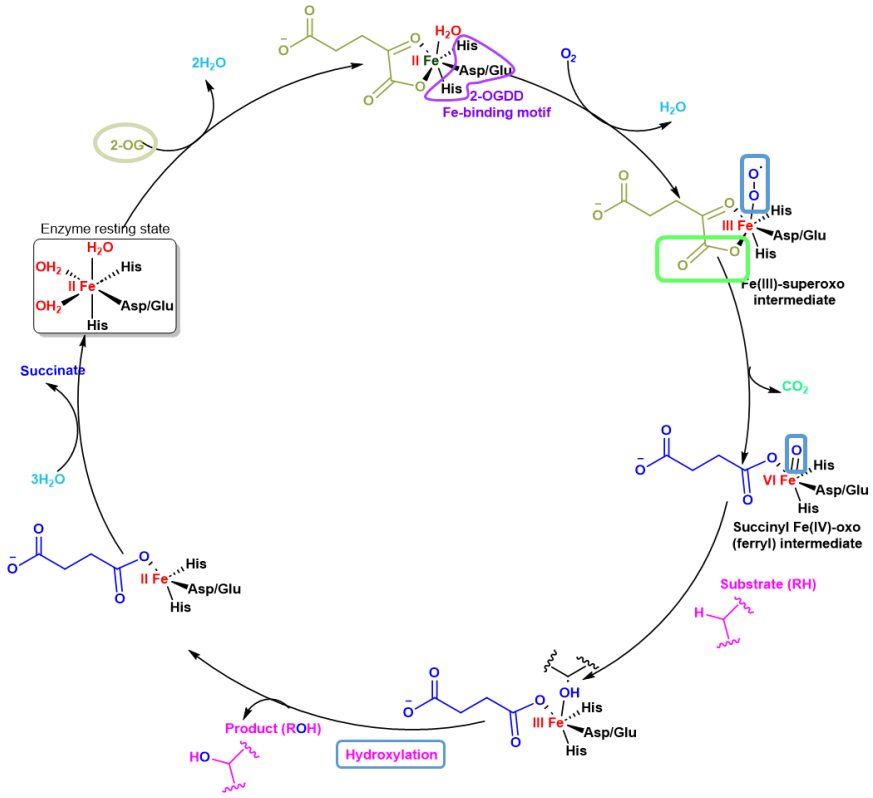

Figure 1.3 Schematic of 2OGDD enzyme-catalyzed hydroxylation, where Fe^{2+}, 2OG, and O_2 drive sequential binding and oxidation, leading to Fe (IV)-oxo formation, substrate hydroxylation, and succinate release as a byproduct (Islam, et al., 2018a).

Figure 1.4 illustrates the modulation of HIF-1α levels and activity in relation to oxygen supply. Under normoxic conditions, proline residues (Pro402/564) of HIF-1α undergo hydroxylation by PHDs, resulting in pVHL-mediated ubiquitination and

subsequent destruction via the 26S proteasome. Additionally, FIH hydroxylates asparagine residue (Asn803), blocking p300/CBP coactivator binding and inhibiting transcriptional activation. In hypoxia, reduced hydroxylase activity stabilizes HIF-1α, allowing dimerization with HIF-1β, recruitment of p300/CBP, binding to HREs, and activation of target gene transcription. This leads to substrate phosphorylation, allowing HIF-1β binds to HIF-1α which activate target gene for the transcription (Drager et al., 2004).

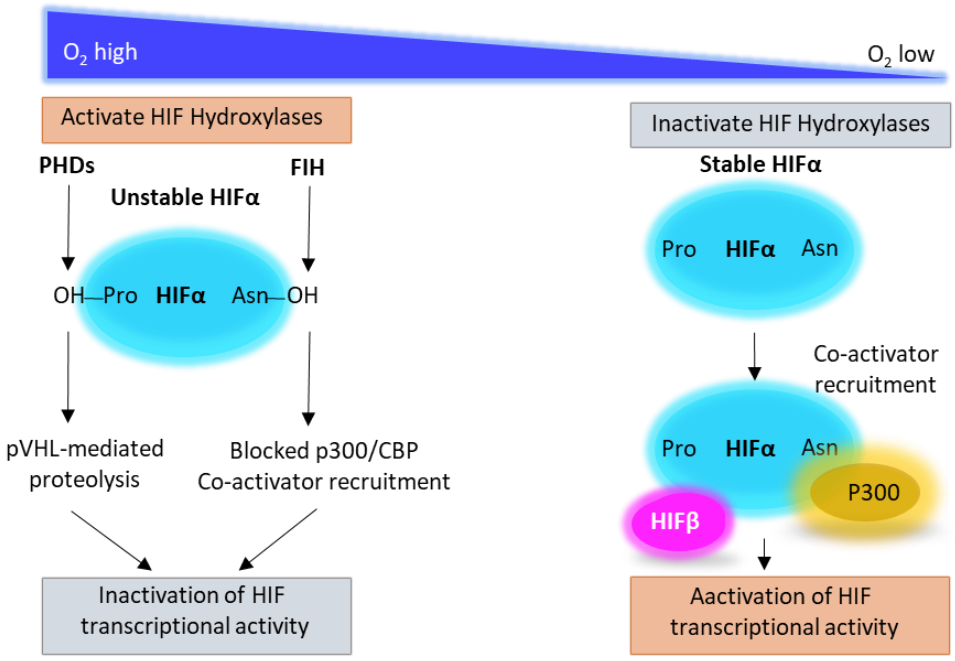

Figure 1.4 HIF-α levels are regulated by oxygen-dependent enzymes prolyl hydroxylase (PHD) and factor inhibiting HIF (FIH). Under normoxic conditions, HIF-1α is hydroxylated at Pro402 and/or Pro564, leading to pVHL-mediated proteasomal degradation. Additionally, hydroxylation at Asn803 by FIH prevents its interaction with the co-activator p300/CBP, suppressing transcriptional activity. In hypoxia, hydroxylase activity is reduced, allowing HIF-α to stabilize, dimerize with HIF-β, bind hypoxia response elements (HREs), recruit p300/CBP, and activate transcription. Adapted from Schofield *et al* (Schofield et al., 2004).

1.5 2-Oxoglutarate (2OG)

2-Oxoglutarate (2OG), also referred to as α-ketoglutarate or 2-oxopentanedioic acid, is a five-carbon molecule containing two carboxyl groups. It serves as an

essential cofactor for PHDs, which regulate the stability of HIF under normoxic conditions (Frost et al., 2021). The relationship between 2OG, PHDs, and HIF is crucial for cellular adaptation to fluctuating oxygen levels, helping to understand the molecular mechanisms behind hypoxia-related diseases and physiological responses. The enzymatic activity of PHD is dependent upon 2OG as a co-substrate (Bailey et al., 2020). Figure 1.5 depicts the co-crystal structure of 2OG with the PHD2 catalytic domain (PDB ID: 3OUJ). The structure indicates that the Fe (II) center is bidentately coordinated by the sp^2 oxygen atoms at C1 and C2 of 2OG. A salt bridge is present between the carboxylate group at C5 and Arg383, whereas a hydrogen bond is established between the of hydroxyl group of Tyr329 phenol. The 2OG binding pocket is also surrounded by a cluster of hydrophobic residues, including Ile327, Ile256, Met299, Leu343, Trp389, Ala301, Ala365, and Val376 (Kim et al., 2015).

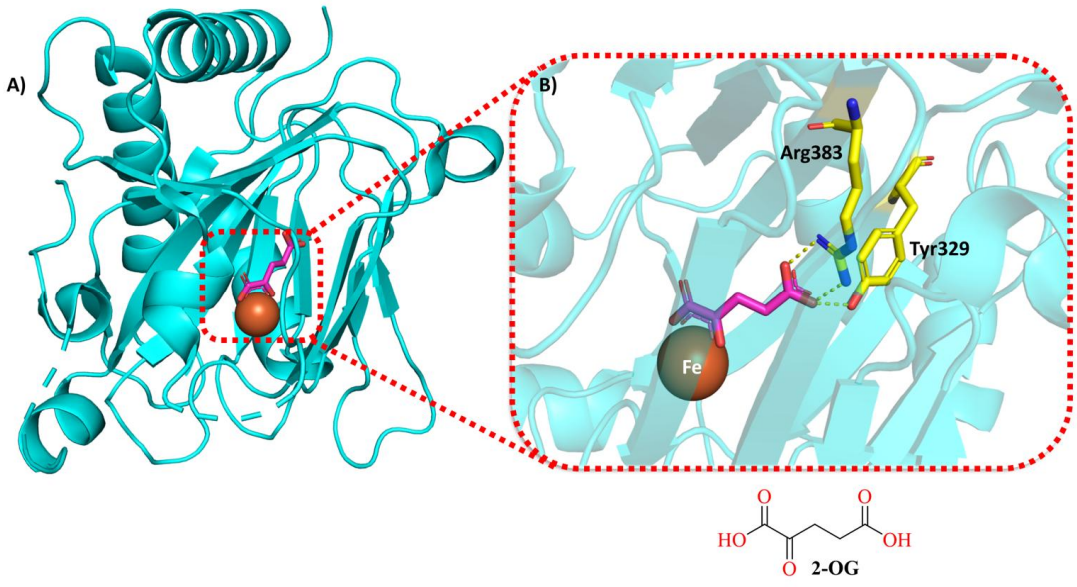

Figure 1.5 2OG binding within the PHD2 active site. A) Structural view of PHD2 accommodating 2OG. B) 3D illustration showing 2OG chelating the Fe (II) center and interacting with key residues Arg383 and Tyr329 (Chowdhury et al., 2020) (PDB: 3OUJ) (Rosen et al., 2010).

1.6 2OG dependent oxygenases (2OGXs)

The 2-oxoglutarate dependent oxygenases (2OGXs) superfamily comprises around 60-70 enzymes in humans, categorized into nucleotide-modifying enzymes, like the ten-eleven translocation methylcytosine dioxygenase (TET) family for DNA demethylation, and protein-modifying enzymes, such as hydroxylases and demethylases. These enzymes share a common catalytic domain with a distorted double-stranded β-helix (DSBH) fold, providing specificity in substrate recognition. 2OGXs use Fe (II), 2OG, and oxygen as cofactors to catalyze oxidative reactions that produce CO_2 and succinate as byproducts as depicted in Figure 1.2 above. Their targets encompass proteins, nucleic acids, lipids, and small molecules, frequently associated with peptides. The most common reactions are hydroxylation, with occasional demethylation via hydroxylation. These enzymes are involved in essential processes such as gene regulation, DNA repair, and hypoxia sensing. Additionally, they play key roles in secondary metabolism, including β-lactam biosynthesis. The biochemical flexibility and specificity of 2OGXs highlight their significance in primary and secondary metabolism, with important implications for human health and therapeutic applications (Islam et al., 2018a).

Crystallographic studies reveal that 2OGXs are defined by a distorted double-stranded β-helix (DSBH) core fold, also known as a jelly-roll motif (Figure 1.6 A). This right-handed, class I DSBH fold is the only one of four theoretical DSBH topologies observed in nature and is shared by cupin and JmjC domain proteins, many of which are 2OGXs. The DSBH fold in 2OGXs was first predicted from studies on isopenicillin N synthase (IPNS), an enzyme structurally homologous to but mechanistically distinct from 2OGXs. Its presence was later confirmed through crystallographic analyses of enzymes such as deacetoxycephalosporin C synthase

(DAOCS), taurine dioxygenase, and clavaminic acid synthase, which also identified subfamily-specific structural features (Markolovic et al., 2016). A structural representation of the prolyl hydroxylase domain from Pseudomonas putida (PPHD), a saprophytic soil bacterium, is also provided for comparison (Figure 1.6 B) (Aik et al., 2015).

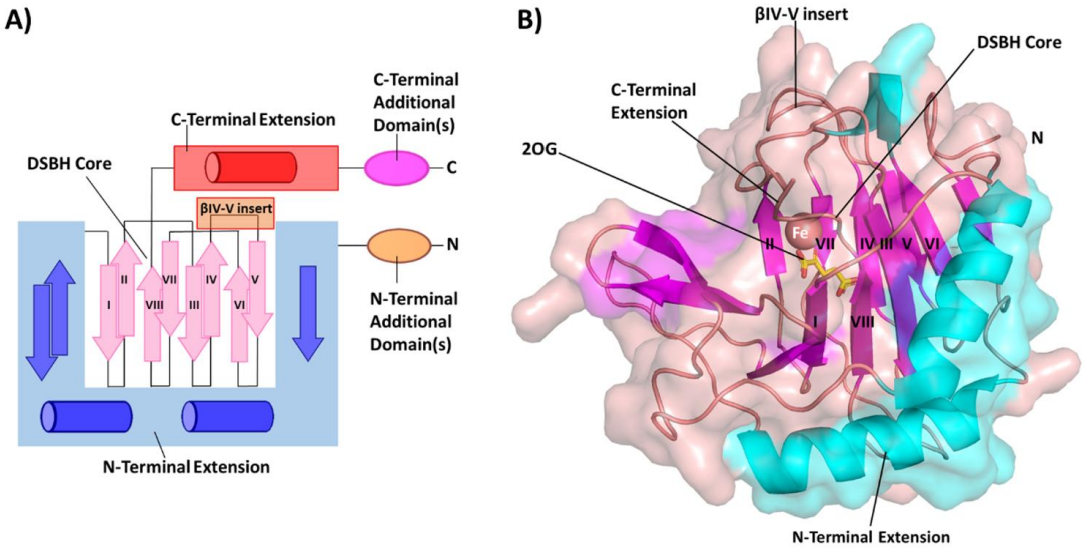

Figure 1.6 Overall structure of the 2OG oxygenases. A) General 2-dimensional topology of a 2OG oxygenase. B) Three-dimensional representation of the 2OG oxygenase fold adapted from a crystal structure of AlkB (PDB ID: 3BIE) (Aik et al., 2015).

The functional classification of 2OGXs is summarized in Figure 1.7, dividing them into nucleotide-modifying enzymes, like TET1-3, and those targeting proteins, including histones and non-histone proteins. A key subgroup, the JmjC-only hydroxylases, catalyze protein hydroxylation, distinct from other JmjC enzymes involved in demethylation. Around 45 of these enzymes function as protein hydroxylases or demethylases, while others target different macromolecules. Table 1.1 details key 2OGX enzymes, highlighting their importance in epigenetic regulation, stress responses, and DNA repair (Fletcher et al., 2020).

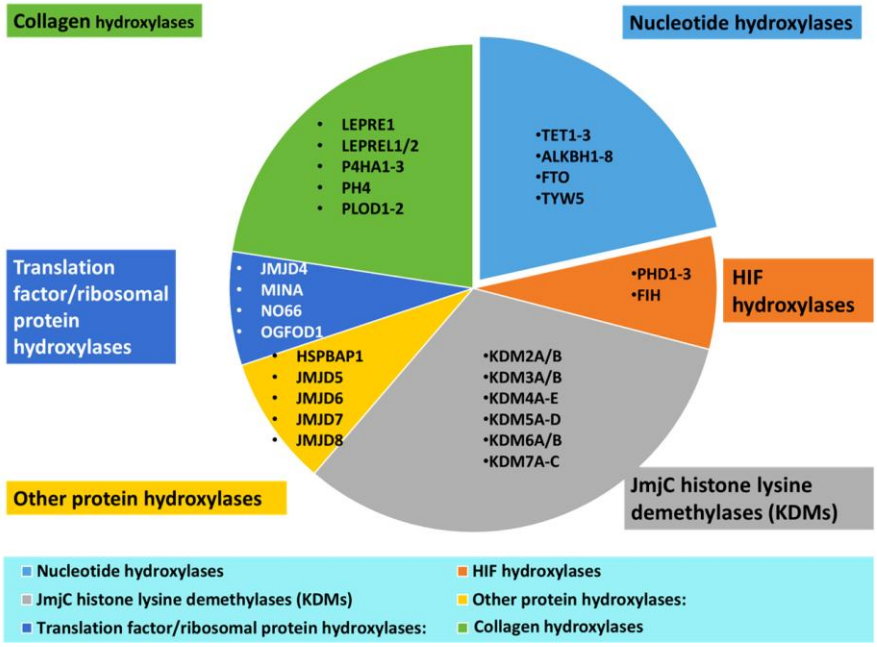

Figure 1.7 Functional grouping of 2OG oxygenases (2OGXs) by substrate type and function: Collagen hydroxylases (green), nucleotide hydroxylases (blue), translation factor/ribosomal protein hydroxylases (light blue), protein hydroxylases (yellow), and JmjC histone lysine demethylases (gray). Key enzymes like TET1-3, PHD1-3, FIH, and various KDMs highlight their roles in nucleotide, HIF, and histone regulation (Fletcher et al., 2020).

Table 1.1 Some of key 2OGXs ,their function and relevant diseases (Fletcher et al., 2020; Yang et al., 2024).

2OGXs/Code	**Members**	**Function**	**Relevant Diseases**
HIF- prolyl hydroxylases (PHDs)	EGLN2 / PHD1, EGLN1 / PHD2, EGLN3 / PHD3	hydroxylation reactions	Cancer, Ischemic disorders Fibrosis Erythrocytosis Metabolic disorders
Jumonji C domain-containing histone lysine demethylases (JmjC-KDMs)	KDM2A-B, KDM3A-C, KDM4A-D, KDM5A-D, KDM6A-C, KDM7A-C	Histone demethylation	Cancer, Neurological disorder
Nucleotide Hydroxylases	ABH1, ABH2, ABH3, ABH4, ABH5, ABH6, ABH7, ABH8, FTO, TET1, TET2, TET3,TYWS	DNA/RNA demethylation	Cancer, Metabolic disease, Neurological disorder
Lysine hydroxylases (LHs)	LH1, LH2, LH3	Collagen post-translational modification	Connective tissue disorder, Fibrosis, Cancer
Collagen Hydroxylases	P4HA1, P4HA2, P4HA3, LEPREL1, LEPREL2, LEPREL3	collagen hydroxylation	Breast cancer, Scurvy, Fibrotic
Translation Factor/Ribosomal Protein Hydroxylases	JMJD4, NO66, MINA, OGFOD1	Translating DNA into proteins	Cancer, Immune dysfunction
Other Protein Hydroxylases	JMJD5, JMJD6, JMJD7, JMJD8, HSPBAP1	//	Neurodegenerative disorder Inflammation Cancer.

This research primarily focused on PHDs, particularly PHD2 and PHD3, where our novel series of carbamoyl derivatives (CDs) (**AMS-1 to AMS-19**) demonstrated good activities for both. PHD2 is the most physiologically significant isoenzyme, contributing to the cellular response to hypoxia, and represents a promising therapeutic target for diseases such as cancer (Sameti et al., 2023). To assess selectivity, we also explored other 2OGXs including factor inhibiting HIF (FIH), Jumonji domain-containing protein 6 (JMJD6) and lysine demethylase 4A (KDM4A). FIH regulates HIF by hydroxylating asparagine residues, complementing PHD's role in oxygen sensing. The KDM and JmjC families, including KDM4A and JMJD6, play central roles in epigenetic regulation and gene expression by modifying histones and other proteins, impacting cellular processes such as transcription and RNA splicing. Collectively, these 2OGXs are critical to understanding cellular responses to environmental stresses and are potential targets for therapeutic interventions (Yang et al., 2024).

1.7 PHD diversity and distribution

PHD enzymes are evolutionarily conserved across a wide range of organisms, reflecting their fundamental biological roles. Although considerable research has concentrated on these enzymes in mammals, especially in cells of mammalian, a comparable hydroxylase has been discovered in the plant *Dictyostelium*. In *Dictyostelium*, the PHD enzyme catalyses the hydroxylation of SKP1, a homolog of the FBOX component of the E3 ubiquitin ligase (McDonough et al., 2006; Van der et al., 2005).

Caenorhabditis elegans and Drosophila respectively possess a single family member within this category. In Drosophila, PHD is known as dHPH (Smith et al.,

2010), while in *C. elegans*, it goes by the name egg laying nine (EGL-9 or EGLN) due to its essential role in egg-laying (Powell et al., 2010). In contrast, mammals exhibit four members within this subfamily, which include PHD1 (EGLN2), PHD2 (EGLN1), PHD3 (EGLN3) and a newly categorized protein referred to as P4HTM (Koivunen et al., 2007; Sears et al., 2011; Trichonas, et al., 2013). Various terms have been used for mammalian homologs, such as HIF-P4H1, HIF-P4H3 and SM-20 for rat PHD3, signifying hydroxylation on the fourth position of proline residues in HIF (Hirsila et al., 2003). It's worth noting that the term 'PHD proteins' aptly describes their functionality as hydroxylases while avoiding exclusive association with HIF substrates, as non-HIF substrates also exist (Fitzpatrick et al., 2016; Fong et al., 2008).

1.7.1 PHD Isoforms

PHD enzymes (PHD1, PHD2, and PHD3) are vital dioxygenases that regulate HIFs, which facilitate cellular adaptation to fluctuating oxygen levels. These enzymes were first identified in 2001 and show a central role in the HIF-mediated hypoxia signaling pathway by hydroxylating HIF-α, marking it for degradation under normoxic conditions. Despite sharing 40%-60% sequence similarity (Figure 1.8), PHD isoforms have evolved distinct physiological roles and tissue-specific expression patterns (Appelhoff et al., 2004). PHD2 is the most ubiquitous isoform, primarily responsible for HIF-α degradation across maximum tissues, while PHD1 is predominantly expressed in the testis, and PHD3 is abundant in the heart (Hoang et al., 2022). These isoforms not only differ in their expression but also in their substrate preferences and physiological functions (Kim et al., 2015; Schödel et al., 2019). For instance, PHD1-deficient mice exhibit enhanced hypoxia tolerance through reprogramming for anaerobic metabolism in skeletal muscle and liver, while PHD2 deficiency results in

embryonic lethality due to critical developmental flaws in the heart and placenta. In contrast, the loss of PHD3 causes a hypofunctional sympathoadrenal system, resulting in lower blood pressure and altered cardiovascular responses. PHD3 is also highly inducible by hypoxia and targets a broader range of substrates, including pyruvate kinase M2 and apelin, highlighting its unique role beyond HIF-α regulation (Demandt et al., 2021).

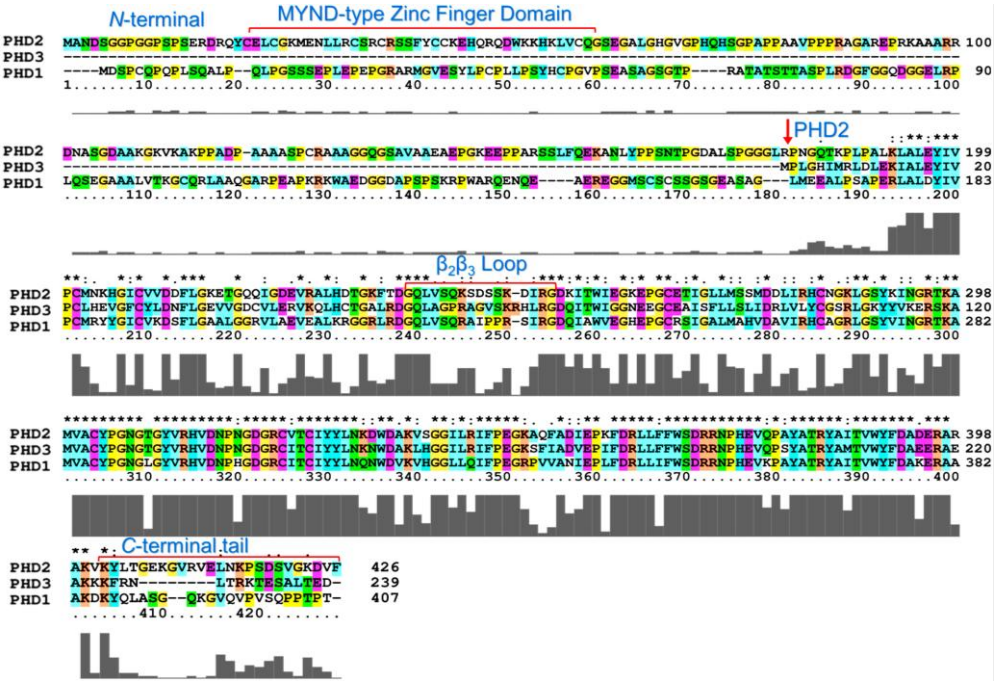

Figure 1.8 Sequence alignment of human PHD1-3 (Flashman et al., 2008). Asterisks (*) denote highly conserved residues, colons (:) indicate moderately conserved residues, and periods (.) represent the least conserved residues. The alignment was generated using ClustalX 2.1 software.

1.7.2 Structural characteristics of PHDs

The whole human PHD1, PHD2, and PHD3 include 407, 426, and 239 amino acid residues, respectively. They share a highly conserved catalytic domain at the C-terminal, displaying around 55% sequence identity (Figure 1.8). This domain contains a double-stranded β-helix (DSBH) core, typical for 2OGXs, which forms the catalytic center essential for their hydroxylase activity (Tarade et al., 2021). The N-terminal

regions, however, are more variable, with PHD3 featuring the shortest N-terminal domain. Crystallographic studies have revealed detailed insights into PHD2's structure, particularly in its complex with Fe (II) and a 2OG analogue, 2-[(4-hydroxy-8-iodoisoquinolin-3-yl)formamido]acetic acid (4HG) inhibitor as shown in Figure 1.9 (PDB ID: 2G19) . The secondary structure shows the DSBH core stabilized by three α-helices and anti-parallel β-strands. The β2/β3 loop, comprising residues 237-254, plays a critical role in substrate recognition and stabilization, undergoing significant conformational changes upon binding the HIF-1α CODD peptide. This structural adaptability facilitates substrate specificity and supports the enzyme's catalytic function. Additionally, PHD2's structure is stabilized by its interaction with Fe (II), which is coordinated within the catalytic center, further enhancing its regulatory role in oxygen sensing (Fiorini et al. 2024).

Together, the differences in expression patterns, substrate preferences, and structural features underscore the complexity of the PHD family and their essential roles in regulating HIF-mediated responses to hypoxia. These enzymes serve as key therapeutic targets for a range of diseases, such as cardiovascular conditions, cancer and metabolic disorders, where impaired hypoxia signaling plays a role in disease progression (Shan et al., 2023).

Mn^{2+} and Ni^{2+} are often used as substitutes for Fe^{2+} in proteins like PHD2 because they are less prone to oxidation and can mimic the coordination geometry of Fe^{2+} (Deng et al., 2013; Holt-Martyn et al., 2019).These ions help maintain the protein's active conformation during crystallization (Handing et al., 2018).

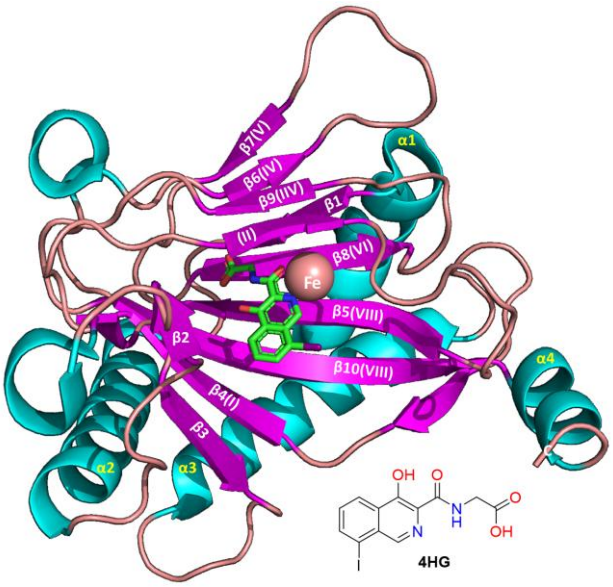

Figure 1.9 Stereo view ribbon diagram depicting the secondary structure of the catalytic domain of PHD2, which is bound to the 2-[(4-hydroxy-8-iodoisoquinolin-3-yl)formamido]acetic acid (4HG) (highlighted in green). The active site Fe (II) ion is represented as an orange sphere (PDB ID: 2G19) (McDonough et al., 2006).

1.7.3 PHDs as oxygen sensors

PHDs play a critical role in sensing oxygen levels within cells (Pientka et al., 2012). They act as oxygen sensors by detecting changes in oxygen concentrations and influencing the stability of HIF-1α (Smith et al., 2015). PHD2, in particular, has garnered attention for its unique characteristics that allow it to function as an efficient oxygen sensor. Researchers have reported that the apparent K_m values for oxygen binding to PHDs are around 240 μM, which is slightly higher than the physiological concentration of dissolved oxygen in the atmosphere (~200 μM) (Fandrey et al., 2006). Interestingly, a studies had suggested a higher K_m value of approximately 1.7 mM for oxygen in PHD2 (Yeoh et al., 2012). Additionally, kinetic investigations have revealed that PHD2 exhibits a slower reaction rate when reacting with oxygen, distinguishing it from other 2OGXs (Domene et al., 2020). These features higher K_m values and slower reaction rates contribute to PHD2' sensitivity to changes in oxygen levels

(Kearney, 2015). Significantly, the importance of PHD2 as a primary human oxygen sensor is emphasized by studies utilizing small interfering RNA (siRNA). Targeted reduction of PHD2 mRNA levels, even under normal oxygen conditions, results in the stabilization of HIF-1α. This underscores the crucial role of PHD2 in regulating HIF-1α stability in an oxygen-dependent manner(Fang et al., 2005). In contrast, downregulation of PHD1 and PHD3 mRNA levels has minimal impact on HIF-1α expression across various cell types. This indicates that PHD2 has a more central role in oxygen sensing compared to the other PHD isoforms (Matoba et al., 2013; Pientka et al., 2012).

The findings are further supported by research demonstrating that PHD2 has the greatest specificity for the primary hydroxylation site of HIF-1α, known as CODD (Yeoh et al., 2012). Moreover, PHD2 is regarded as the ancestral variant of the PHD (EGLN) gene family, as evidenced by research on the nematode *C. elegans* (Ma et al., 2022).

1.8 Problem statement

Hypoxia-related diseases, including anemia, chronic kidney disease (CKD), and cardiovascular diseases (CVDs) are among the leading contributors to global mortality. Cardiovascular illnesses contribute for around 17.9 million deaths per year, constituting 32% of total global mortality (Luo et al., 2024). Anemia, which affects around 15% of the global population (Hess et al., 2023), further compromises oxygen delivery, leading to a 40% rise in cardiovascular complications and a 30% increase in mortality risk, as highlighted by the 2022 WHO statistics (Bianchi et al., 2024). Similarly, CKD represents a growing global health crisis, with renal hypoxia impacting an estimated 850 million people worldwide and approximately 700 million

diagnosed with CKD. The global rate of chronic kidney disease (CKD) increased by 33% from 1990 to 2017, with the most significant rise observed in countries with low or middle incomes, notably China and India (Francis et al., 2024).

To address these pressing issues, a promising therapeutic strategy lies in activating hypoxia inducible factors (HIF) by inhibiting prolyl hydroxylase domain (PHD) enzymes. This method stabilizes HIF and triggers protective pathways under hypoxic conditions. However, existing PHD inhibitors present significant limitations, including toxicity, thromboembolic risks, and high costs, underscoring the urgent need for safer and more effective alternatives (Locatelli et al., 2022).

This study aims to address the limitations of current PHD-targeted therapies by using computer-aided drug design (CADD) to develop novel inhibitors. Objectives include elucidating the molecular basis of PHD inhibition, designing compounds based on structure-activity relationships (SAR), and evaluating their therapeutic potential. To achieve this, natural, synthetic, and purchasable compounds including those from the REAL database of EnamineStore will be screened. The findings are expected to advance the understanding of hypoxia-related diseases and aid in the development of innovative treatments. *In silico* methods such as virtual screening (VS), induced fit docking (IFD), and molecular dynamics simulations (including RMSD, Rg, RMSF, hydrogen bond analysis, MM/GBSA calculations, and SASA) are employed to predict the binding affinities and inhibitory potential of top-performing compounds (Sayaf et al., 2024). Additionally, AutoQSAR modeling is used to enhance the predictive accuracy of compound efficacy.

In addition, a novel series of carbamoyl derivatives (CDs) was evaluated using IFD. These compounds were selected based on previous findings that 3-

carbamoylpropanoic acid (3CA) derivatives derived from succinic anhydride exhibit potent *in vitro* PHD2 inhibition (e.g., F2a, IC_{50} = 2.24 μM; F2b, IC_{50} = 1.32 μM) (Chong et al., 2021), highlighting their potential as promising scaffolds for further PHD2 inhibitor development. The incorporation of maleic and glutaric anhydrides in the present study was intended to broaden structural diversity and explore structure–activity relationships (SAR) with the goal of enhancing both inhibitory potency and pharmacokinetic properties.

1.9 Research objectives

To address the above concerns and optimize the discovery of novel PHD inhibitors, the following aims are covered in this thesis:

1. To explore natural and synthetic compounds from diverse databases, including AnalytiCon Discovery Natural Product (NP), AfroDb Natural Products, Herbal Ingredients Targets, HIM-Herbal Ingredients in vivo Metabolism, IBScreen NP, Indofine NP, and Naturally Occurring Plant based Anticancer Compound-Activity-Target Database (NPACT) as well as regional African databases (East, North, North-East, and South Africa) and the Comprehensive Marine Natural Products Database (CMNPD), for the discovery of promising PHD inhibitors using *in silico* approaches including molecular docking and molecular dynamics simulations.
2. To investigate the inhibitory potential of REAL database compounds from EnamineStore against PHDs through integrated *in silico* and *in vitro* assays.
3. To design, synthesize, and evaluate a novel series of carbamoyl derivatives (CDs) as potential PHD inhibitors through integrated *in silico* modeling and *in vitro* biological analysis.

1.10 Scope of study

To identify potential PHD2 inhibitors, a comprehensive dataset comprising 147,583 compounds in 3D-SDF format was compiled from a wide range of publicly available and commercially accessible sources. These included the East African Natural Products Database (EANPDB), North African Natural Products Database (NANPDB), North East African Natural Products Database (NEANPDB), South African Natural Products Database (SANCDB), Comprehensive Marine Natural Products Database (CMNPD), Naturally Occurring Plant-based Anti-cancer Compound-Activity-Target (NPACT), Indofine Natural Products, InterBioScreen (IBScreen), AfroDb, AnalytiCon Discovery Natural Products (NP), Herbal Ingredients Targets, and Herbal Ingredients in vivo Metabolism (HIM). In addition, purchasable small-molecule analogues from the REAL database of EnamineStore were included to enhance the translational potential of identified hits. The selection of compounds from these databases was guided by multiple criteria, including natural product origin, structural diversity, reported or predicted bioactivity, physicochemical properties compliant with Lipinski's Rule of Five (RO5), and potential relevance to hypoxia-related targets such as prolyl hydroxylase domain (PHD) enzymes. This curated and diverse compound library served as the foundation for subsequent virtual screening and molecular modeling studies.

To ensure drug-likeness, we applied Lipinski's Rule of Five (RO5) using the FAF Drugs4 web server. Compounds that passed the rule were prepared and subjected to screening against the PHD2 active site using ligand-based and structure-based techniques. The compounds explored interact with key residues such as Arg383, Tyr329, Tyr303, and Asp254, while also forming bidentate coordination with the iron (II) ion in the PHD2 active site.

In silico drug design techniques, including binding free energy estimation and binding pose analysis based on crystallographic data from the PDB, were employed. Molecular dynamics simulations, including RMSD, Rg, RMSF, hydrogen bond analysis, MM/GBSA calculations, and SASA, were conducted by Dr. Abbas Khan at Qatar University and Shanghai Jiao Tong University.

The synthesized CDs were analyzed using multiple spectroscopic techniques, such as Fourier-transform infrared spectroscopy (FT-IR) and Nuclear magnetic resonance (NMR) (1D and 2D), at the School of Chemical Sciences, Universiti Sains Malaysia.

Part of this research (Chapter 7) was conducted under the supervision of Professor Dr. Christopher J. Schofield from the Schofield Group (CJS), Chemistry Research Laboratories (CRL), University of Oxford. High-resolution mass spectrometry (HRMS) of the CDs was analysed with Thomas Corner, and the expression of PHD3 protein was investigated with Dr. Eidarus Salah and Parsa Jabbary. Furthermore, compounds purchased from the REAL database of EnamineStore and successfully synthesized CDs were tested for inhibitory potency against PHD2, PHD3, and FIH, using the Solid phase extraction mass spectrometry (SPE-MS), conducted together with Dr. Anthony Tumber and Dr. James Holt-Martyn, while CPMG NMR binding studies for **AMS-12** from synthesized CDs were performed with Sam Atwal from CJS at CRL, University of Oxford.

This comprehensive approach aims to identify potent and selective PHDs inhibitors with therapeutic potential for treating hypoxia-related diseases.

CHAPTER 2

LITERATURE REVIEW

This review begins with a historical overview of HIF and PHD enzymes, tracing their discovery and evolution, and then delves into the genes associated with HIF. It explores the therapeutic benefits of activating HIF through PHD inhibition, highlighting its potential to impact disease treatment and management. The role of PHDs in drug discovery is also examined, emphasizing their potential as promising targets for novel therapeutics. A significant portion of the review focuses on reported PHD inhibitors, categorized by their chemical structure and mechanisms of action. This includes a detailed analysis of 2-oxoglutarate (2OG) analog inhibitors, such as: (i) carboxylic acid-based PHD inhibitors (CABPIs) like N-oxalylglycine (NOG) and its derivatives (quinoline, pyridine, benzimidazole), and (ii) non-carboxylic acid-based inhibitors (NCABPIs), including quinazolines, hydroxypyridones, and spiroindolones, along with non-conventional PHD inhibitors (NCPIs) like phenolic derivatives and pyrithione zinc complexes.

The review also discusses the importance of amide bond formation in PHD2 inhibitor design, highlighting the role of amides in drug design and recent advances in their synthesis. Finally, the applications and limitations of CADD are explored, emphasizing its crucial role in discovering and developing PHD inhibitors. This literature review offers an extensive overview of the existing research on PHD enzymes and their inhibitors, establishing a foundation for future investigation and advancement in this evolving field of biomedical science.

2.1 Historical overview of HIF research

The exploration of HIFs has profoundly enhanced our understanding of cellular responses to low-oxygen conditions. The chronological evolution of HIF research, highlighting key milestones is summarized in Table 2.1. This scientific journey began in 1991 with the groundbreaking identification of HIF-1 as a member of the basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) superfamily. This discovery, led by Semenza GL and colleagues, established the pivotal role of HIF-1 in regulating genes associated with angiogenesis, erythropoiesis, and glucose metabolism (Semenza, et al., 1991). Subsequent progress in 1995 by Wang GL and collaborators elucidated the dimerization and transactivation properties of HIF-1, providing key insights into the molecular mechanisms of HIF-mediated gene expression (Wang et al., 1995). In 1999, focus turned to the VHL tumour suppressor protein, which was demonstrated to facilitate the proteasomal degradation of hydroxylated HIF-1α in normoxic circumstances. This critical finding by Maxwell PH and his team underscored the significance of HIF-1α regulation in maintaining cellular oxygen homeostasis (Maxwell et al., 1999). The same year, the overexpression of HIF-1α in various human cancers further highlighted the role of HIF dysregulation in tumorigenesis (Maxwell et al., 1999). Early 2000s marked a major breakthrough with the identification of PHD enzymes as key regulators of HIF stability. In 2001, researchers identified that PHD enzymes hydroxylate particular proline residues in HIF-α subunits, resulting in their breakdown and modulating HIF activity (Ivan et al., 2001). Further advancements in 2002 demonstrated that HIF-1α transcriptional activity could be negatively regulated, shedding light on the intricate control mechanisms governing HIF-mediated gene expression (Wang et al., 1995). In 2003, studies expanded the understanding of cell-specific hypoxic responses, revealing that different cell types exhibit distinct HIF