SYNTHESIS AND CHARACTERIZATION OF POTENTIAL HUMAN HYPOXIA INDUCIBLE FACTOR (HIF) PROLYL HYDROXYLASE DOMAIN 2 (PHD-2) INHIBITORS

TOH LEE ROY

UNIVERSITI SAINS MALAYSIA

2021

SYNTHESIS AND CHARACTERIZATION OF POTENTIAL HUMAN HYPOXIA INDUCIBLE FACTOR (HIF) PROLYL HYDROXYLASE DOMAIN 2 (PHD-2) INHIBITORS

by

TOH LEE ROY

Thesis submitted in fulfilment of the requirements
for the degree of
Master of Science

April 2021

ACKNOWLEDGEMENT

First, I would like to express my sincere gratitude and appreciation to my supervisor, Dr. Yeoh Kar Kheng for his persistent guidance, inspiration and support that made this study a success. I have received a lot of supports and valuable advices during this study.

I would like to acknowledge Universiti Sains Malaysia for offering me the facilities throughout my study. My sincere thanks also go to all the technical staffs of the School of Chemical Sciences, USM especially Mr. Nizam, Mrs. Wan Zulilawati, Mrs. Asma, Mr. Nazeef, Mr. Ramlee and Miss Alia, who helped during many stages of this study.

I wish to thank my friends and colleagues, Ms. Chong Mui Phin, Mr. Maadh, Mr. Suhaib, Dr. Zuhair and Wong Kok Tong who had encouraged and provided many valuable comments and suggestions during my study.

Finally, my sincere appreciation to my dearest family members who have been very supportive and encouraging throughout my study. They have supported me in every way possible during ups and downs in my life. My parents especially have been selfless in giving me the best of everything. Words cannot express my sincere gratefulness for their constant encouragement and love.

TABLES OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF SCHEMES

Page

LIST OF ABBREVIATIONS AND SYMBOLS

1D	1 dimension
2D	2 dimensions
2OG	2-oxoglutarate
α-KG	α-ketoglutarate
Aa	Amino acid
ACD	Anemia of chronic disease
Acetone-d6	Deuterated acetone
Ala	Alanine
Arg383	Arginine residue 383
Asn803	Asparagine residue 803
Asp201	Aspartic acid residue 201
Asp315	Aspartic acid residue 315
bHLH	Basic helix-loop-helix
BIQ	Bicyclic isoquinoline
CADD	Computer aided drug design
CKD	Chronic kidney disease
CODD	C-terminal oxygen-dependent degradation domain
COSY	Correlated spectroscopy
C-TAD	C-terminal transactivation domain
δ	Chemical shift value
d	doublet
DABCO	1,4-Diazabicyclo[2.2.2]octane
°C	Degree Celsius
DCM	Dichloromethane
DFO	Desferrioxamine

DIPEA	Diisopropylethylamine
DMOG	Dimethyl N-oxalyl-glycine
DMSO	Dimethylsulfoxide
DMSO-d6	Deuterated dimethylsulfoxide
DNA	Deoxyribonucleic acid
EPO	Erythropoietin
ESI	Electrospray ionization
EtOAc	Ethyl acetate
FAS	Ferrous ammonium sulphate
FIH	Factor inhibiting HIF
FT-IR	Fourier transform infrared spectroscopic
g	gram
HIF	Hypoxia inducible factor
His199	Histidine residue 199
His279	Histidine residue 279
His313	Histidine residue 313
His374	Histidine residue 374
HMBC	Heteronuclear multiple bond correlation
HRMS	High resolution mass spectrometry
HRP	Horseradish peroxidase
HSQC	Heteronuclear single quantum correlation
IC_{50}	Half-maximal inhibitory concentration
ID	Identification document
IgG	Immunoglobulin G
Ile256	Isoleucine residue 256
J	Coupling constant
JmjC	Jumonji

KBR	Potassium bromide
L-AA	L-Ascorbic acid
LLC	Limited liability company
m.p.	Melting point
m/z	Mass to charge ratio
MeOH	Methanol
Met299	Methionine residue 299
mg	milligram
ml	Milliliter
mmol	millimole
Mol	mole
MS	Mass spectrometry
N	Normality
NMR	Nuclear magnetic resonance
NODD	N-terminal oxygen-dependent degradation domain
NOG	N-oxalylglycine
ODD	Oxygen-dependent degradation domain
p	Pentet
PAGE	Polyacrylamide gel electrophoresis
PAS	Per-ARNT-Sim
PBS	Phosphate-buffered saline
PDB	Protein data bank
pH	Potential of hydrogen
PHD	Prolyl hydroxylase domain
ppm	Part per million
Pro402	Proline residue 402
Pro564	Proline residue 564

PVDF	Polyvinylidene difluoride
pVHL	Von Hippel-Lindau tumor suppressor protein
q	quartet
QTOF	Quadrupole-time-of-flight
RBC	Red blood cells
RCSB	Research collaboratory for structural bioinformatics
R_f	Retention factor
RF-MS	RapidFire mass
RMSD	Root mean square deviation
s	singlet
SDS	Sodium dodecyl sulfate
SP	Standard precision
SPE	Solid phase extraction
t	triplet
TLC	Thin layer chromatography
Trp258	Tryptophan residue 258
Trp389	Tryptophan residue 389
μL	Microlitre
UPLC	Ultra-performance liquid chromatography
VEGF	Vascular endothelial growth factor

LIST OF APPENDICES

SINTESIS AND PENCIRIAN PERENCAT FAKTOR PENDORONG HIPOKSIA MANUSIA (HIF) PROLIL HIDROKSILASI 2 (PHD-2) YANG BERPOTENSI

ABSTRAK

Perencatan farmakologi enzim prolil hidroksilase domain (PHD) telah disarankan sebagai kaedah alternatif untuk menaikan peraturan faktor induktif hipoksia (HIF) dan berfungsi sebagai kaedah terapi untuk penyakit seperti anemia dan penyakit kardiovaskular. Kajian ini bertujuan untuk menilai lima siri sebatian: asid 2H-chromene-3-karbosilik (**A1 - A3)**, triazin (**B)**, pirimidin (**C)**, benzenesulfonamid (**D1 - D4)**, dan benzoxazolamine (**E1 - E3**) sebagai inhibitor perencat PHD-2. Mod pengikatan dan tenaga bebas **A1 - E3** pertama kali dinilai menggunakan kajian sambungan molekul. Hasil dok menunjukkan bahawa semua sebatian yang diuji mampu mengikat ke tapak aktif PHD-2 secara bidentate dan membentuk interaksi jambatan garam dengan residu asid amino Arg383 selain daripada menunjukkan tenaga bebas pengikatan yang istimewa. Sebatian kemudian disintesis dan dicirikan menggunakan FT-IR, HRMS, NMR ^{1}H, ^{13}C NMR dan 2D NMR untuk mengesahkan struktur. **A1 - E3** kemudian disaring untuk potensi penghambatan mereka terhadap PHD-2 menggunakan PHD-2 RapidFire assay. Hasil penghambatan menunjukkan bahawa sebatian **E1** adalah perencat PHD-2 yang kuat, dengan nilai IC_{50} 17,45 μM. Sebaliknya, etil ester **E2** dan **E3** disintesis dan diuji dalam kajian berasaskan sel. Namun, mereka tidak menunjukkan kemampuan untuk menginduksi HIF-1α sebagai petunjuk perencat PHD-2 selular.

SYNTHESIS AND CHARACTERIZATION OF POTENTIAL HUMAN HYPOXIA INDUCIBLE FACTOR (HIF) PROLYL HYDROXYLASE DOMAIN 2 (PHD-2) INHIBITORS

ABSTRACT

Pharmacological inhibition of prolyl hydroxylase domain (PHD) enzymes have been suggested as an alternative method to upregulate hypoxia inducible factor (HIF) and serve as a therapeutic method for diseases such as anemia and cardiovascular disease. This study aims at evaluating five series of compounds: 2H-chromene-3-carboxylic acids (**A1 – A3)**, triazine (**B)**, pyrimidine (**C)**, benzenesulfonamides (**D1 – D4)**, and benzoxazolamine (**E1 – E3**) as PHD-2 inhibitors. The binding modes and free energies of **A1 – E3** were first evaluated using molecular docking studies. The docking results demonstrated that all the tested compounds were capable of binding to the PHD-2 active site in a bidentate manner and forming salt bridge interaction with amino acid residue Arg383 apart from displaying preferential free energies of binding. The compounds were subsequently synthesized and characterized using FT-IR, HRMS, ^{1}H NMR, ^{13}C NMR and 2D NMR to confirm the structures. **A1 – E3** were then screened for their inhibitory potencies against PHD-2 using a PHD-2 RapidFire assay. The inhibitory results revealed that compound **E1** was a potent PHD-2 inhibitor, with IC_{50} value of 17.45 μM. On the other hand, ethyl ester **E2** and **E3** were synthesized and tested in cell-based study. However, they showed no ability to induce HIF-1α as an indicator of cellular PHD-2 inhibition.

CHAPTER ONE

INTRODUCTION

1.1 Hypoxia inducible factors (HIFs) and HIF hydroxylases

Hypoxia is a condition whereby there is a lack of oxygen in body tissues with reduced oxygen levels as low as or less than 2% of oxygen concentration[1]. Hypoxia inducible factors (HIFs) are heterodimeric transcription factors composed of α and β subunits[2,3]. There are three α subunits [HIF-1α, HIF-2α and HIF-3α] and three β subunits (HIF-1β, HIF-2β and HIF-3β)[4,5].

HIF-1α is the most extensively studied and was first identified by Semenza, Wang and co-workers[2,6–10]. HIF-1α possess a full central oxygen-dependent degradation domain (ODD) which is absent in HIF-β[11,12]. There are two transactivation domains exist within ODD of HIF-1α, N-terminal named NODD containing Pro402 and C-terminal named CODD containing Pro564[13–15]. which enable the degradation of HIF-1α. HIF-1α is also hydroxylated at an asparagine residue (Asn803 in HIF-1α) at C-terminal transactivation domain (C-TAD)[12].

HIF-1α is ubiquitously expressed[1,11,16,17]. Expression of HIF-1α is induced by hypoxia, tissue ischemia or regulated by cellular oxygen tension and hence HIF-1α is a regulatory protein[2,8,18–22]. HIF-1β is a constitutive nuclear protein which constitutively expressed regardless of changes in oxygen availability[23]. Anatomy of HIF-1α is shown in **Figure 1.1**.

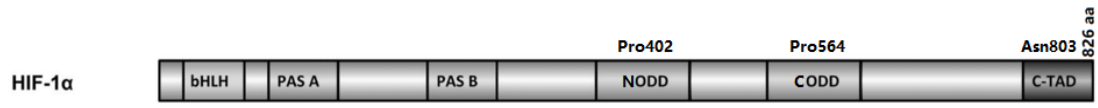
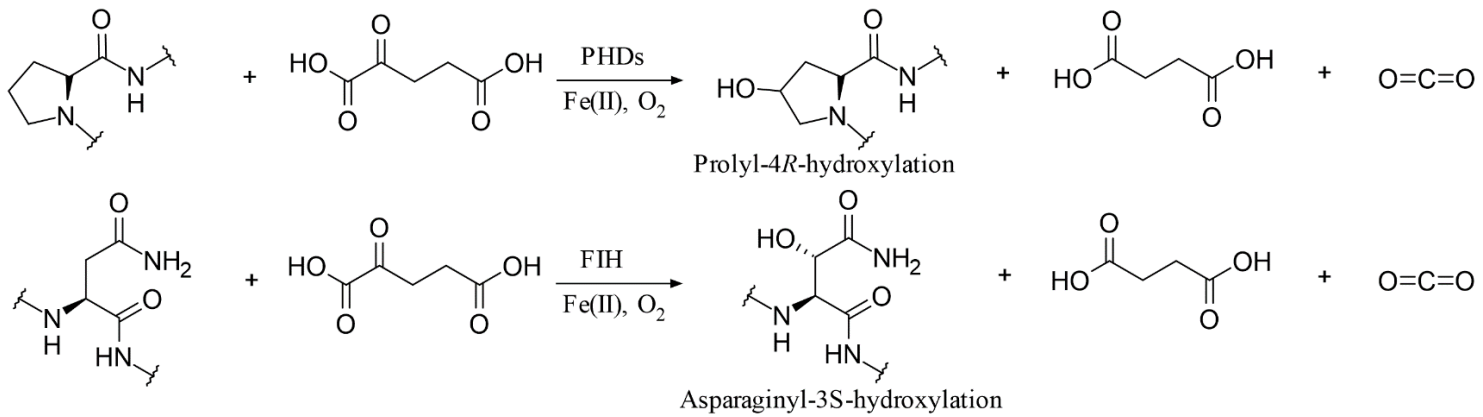


Figure 1.1: Anatomy of HIF-1α subunit. Basic helix-loop-helix (bHLH), Per-ARNT-Sim (PAS), N-terminal oxygen-dependent degradation domain (NODD), C-terminal oxygen-dependent degradation domain (CODD), C-terminal transactivation domain (C-TAD) and number of amino acid (aa) residues are shown[24].

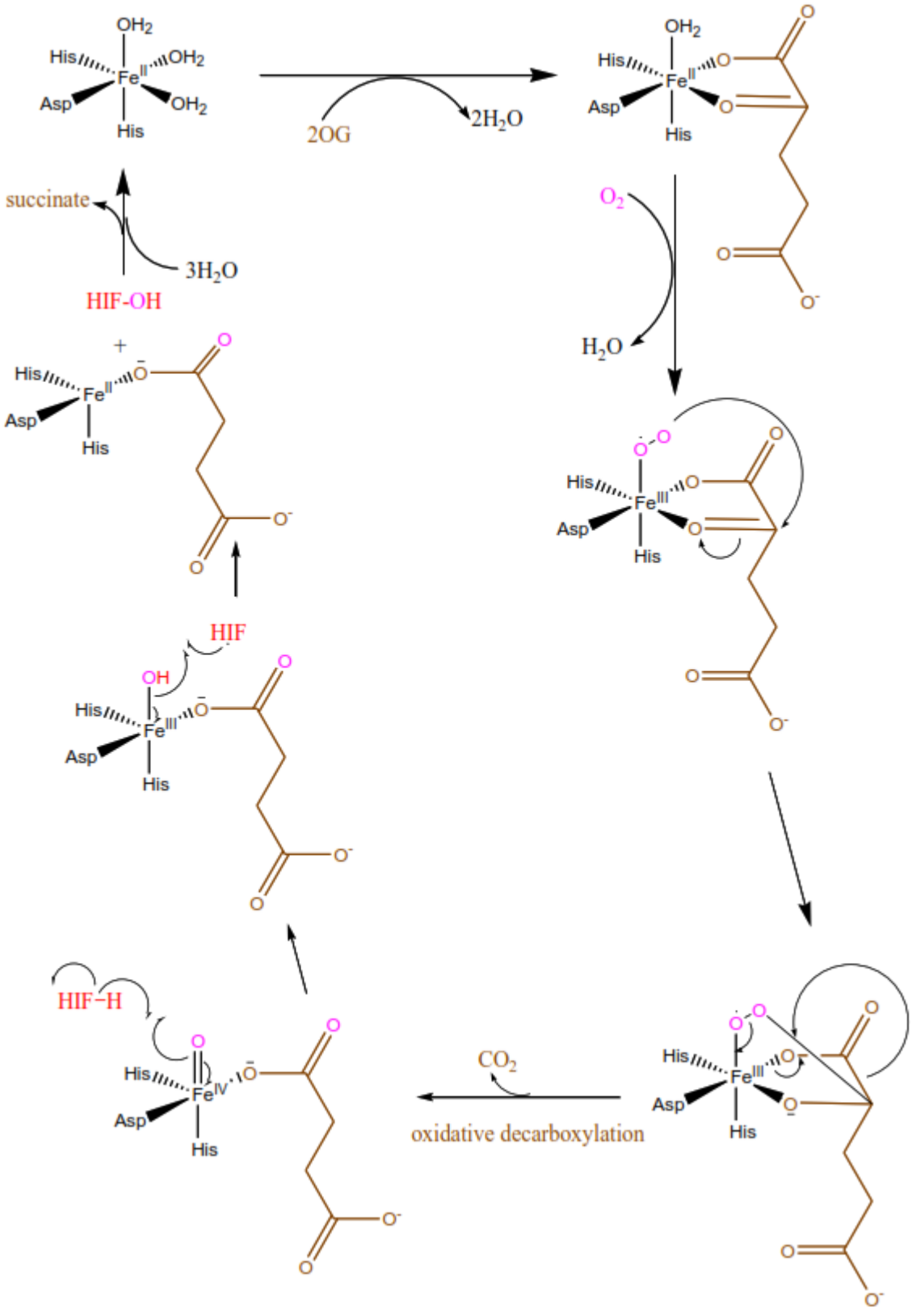
Despite HIF-1α is ubiquitously expressed, HIF-1α protein level is regulated by prolyl hydroxylase domain (PHD) enzymes. There are three members in the conserved proline hydroxylase enzyme family known as PHD-1, PHD-2 and PHD-3[25]. Recently, putative PHD-4 has been reported[26]. PHD-2 has the highest activity towards the primary hydroxylation site of HIF-1α and believed to be the main isoform involved in regulating HIF-1α[27,28].

PHDs belong to an extensive family of 2-oxoglutarate (2OG) dependent, non-haem dioxygenases which utilize oxygen as the co-substrate in hydroxylation[29–31]. Hydroxylation is halted during hypoxia due to the low concentration of oxygen present which then restores HIF stability and activity[29,30]. Therefore, PHDs are regarded as dioxygen sensors.

Beside regulation by PHDs, HIF-1α is also regulated by Factor inhibiting HIF (FIH). FIH is a member of new sub-family of iron- and 2OG dependent dioxygenases and one of jumonji (JmjC) transcription factors[32,33]. FIH contains one iron (II) ion coordinated octahedrally by side chains of His199, Asp201 and His279. The HIF hydroxylation reactions are shown in **Scheme 1.1** and the general mechanism of 2OG deoxygenases is shown in **Scheme 1.2**.



Scheme 1.1: The hydroxylation reactions catalyzed by HIF prolyl hydroxylase domains (PHDs) and Factor inhibiting HIF (FIH)[30].



Scheme 1.2: The proposed mechanism of action of 2OG dependent oxygenases[34].

1.2 HIF signaling pathways

HIF have two different signaling pathways which occur during normoxia and hypoxia. During normoxia, PHDs hydroxylate two critical proline residues (P402 and P564) of HIF-1α in its oxygen-dependent degradation domains (ODD)[35]. The proline hydroxylation enables binding of ODD with von Hippel-Lindau tumor suppressor protein (pVHL) to undergo ubiquitination and proteasomal degradation[12,36,37]. HIF-1α has a half-life of <5 min and is negatively regulated during normoxia while the constitutive intracellular levels of HIF-1α is almost undetectable[2,38–41].

Besides hydroxylation of proline residues, HIF-1α is also hydroxylated at the Asn803 residue by Factor inhibiting HIF (FIH)[35,42,43]. Hydroxylation of Asn803 residue prevents interaction of HIF-1 with p300 and CBP transactivators for transcription of target genes. Both hydroxylation by PHDs and FIH require oxygen, 2-oxoglutarate and iron (II) ion and generates succinic acid and carbon dioxide as byproducts[13,14,44,45].

During hypoxia, oxygen concentration is low, activity of PHDs and FIH are reduced. HIF-1α is then stabilized with reduced binding to pVHL[12,46]. Next, availability of HIF-1α to ubiquitination and proteasomal degradation is reduced[46–48]. Therefore, HIF-1α hydroxylation activity is reduced and activity of HIF-α subunit is stabilized. HIF-1α then translocates to the nucleus and dimerizes with HIF-1β subunit to form heterodimeric activated HIF-1 complex[39,49,50]. HIF-1 complex then binds to hypoxia responsive elements (HREs) in their target genes to activate various target genes important in adaption to hypoxia such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO)[1,6,12,22,23,35,41,51–53]. The HIF signaling pathway during normoxia and hypoxia is shown in **Figure 1.2**.

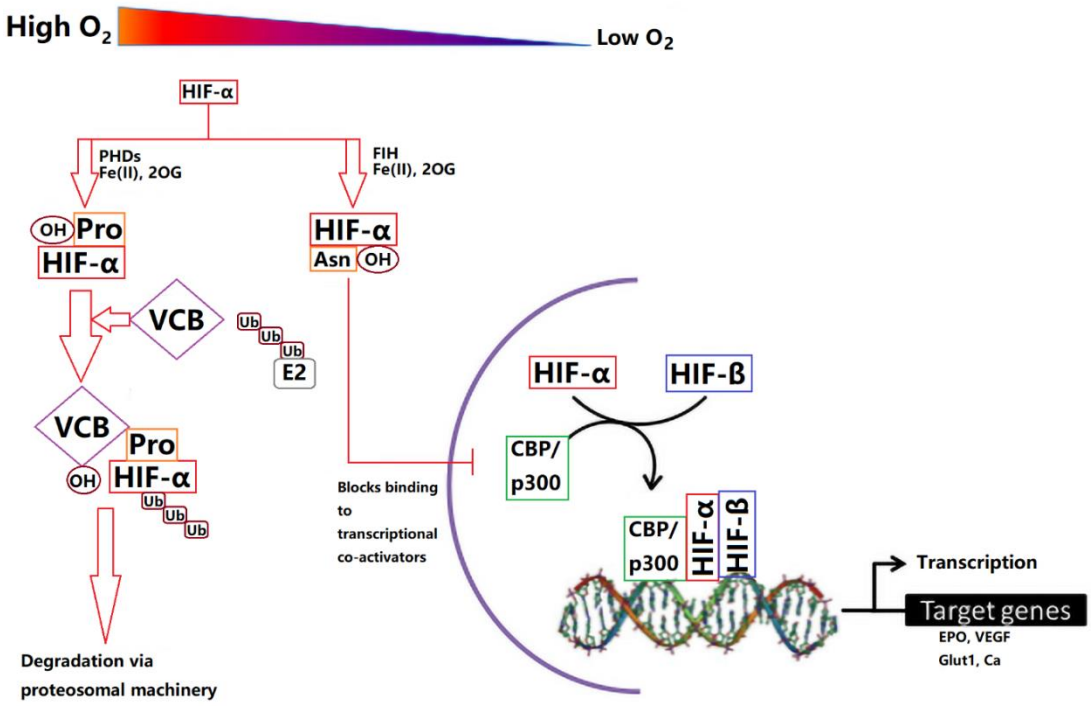


Figure 1.2: HIF signaling pathway during normoxia and hypoxia[54].

1.3 Problem statement

Inhibition of PHDs have been suggested as an alternative method to upregulate HIF to serve as a treatment to the diseases such as anemia and ischemic diseases[55]. Anemia is considered as a critical disease caused by several factors such as chronic kidney disease (CKD)[56] and anemia of chronic disease (ACD)[57]. However, the current treatment either cause fluctuation of iron and red blood cells (RBC) or high treatment cost. The discovery of new small molecule inhibitors will make treatment less costly and less dependent on administration of either recombinant erythropoietin or red blood cells. However, current small molecule inhibitors are not effective to replace administration of either recombinant erythropoietin or red blood cells. In this study, different classes of potential PHD-2 inhibitors compounds have been synthesized and tested as potential PHD-2 inhibitors.

1.4 Research objectives

The research objectives:

1. To study the binding affinity of 2H-chromene-3-carboxylic acid, triazine, pyrimidine, benzenesulfonamide and benzoxazolamine as potential PHD-2 inhibitors using computer aided technique (molecular docking)
2. To synthesize and characterize the above mentioned compounds using various spectroscopic methods
3. To study the inhibitory potencies of the synthesized compounds using PHD-2 RapidFire assay
4. To investigate the cell-based studies of the benzoxazolamine analogues to induce HIF-1α as an indicator of cellular PHD-2 inhibition

1.5 Scopes of study

This study focuses on the synthesis, characterization and inhibitory studies of different classes of compounds (2H-chromene-3-carboxylic acid, triazine, pyrimidine, benzenesulfonamide and benzoxazolamine) against human HIF prolyl hydroxylase domain 2 (PHD-2). The structural design was based on the interactions of these compounds with the PHD-2 enzyme including hydrogen bonding with Arg383 residue and bidentate coordination with the active site iron (II) ion. *In silico* computer aided drug design technique was implemented in the early stages to screen the designed compounds based on Lipinski's Rules, estimation of the free energy of binding and binding pose of the compounds in the active site of PHD-2 enzyme based on the crystallographic structure available from www.pdb.com. Lipinski's Rule of Five states that orally active drug must have less than six hydrogen bond donors, less than eleven hydrogen bond acceptors and below molecular weight of 500 g/mol. Compounds

which showed good binding affinity in molecular docking studies were further synthesized and characterized using various spectroscopic techniques such as nuclear magnetic resonance (NMR) (1D and 2D), mass spectrometry (MS) and Fourier transform infrared spectroscopic (FT-IR). The inhibitory potencies of the compounds were screened using RapidFire PHD-2 hydroxylation assay. The assay was carried out by Dr. Martin Abboud at Chemical Research Laboratory, University of Oxford. The cell-based studies of the benzoxazolamine analogues to induce HIF-1α as an indicator of cellular PHD-2 inhibition was carried out by Chan Yan Ying at Dr. Andrew Chan Mun Chiang's lab in Department of Molecular Medicine, University of Malaya.

CHAPTER TWO

LITERATURE REVIEW

2.1 HIF target genes

Hypoxia is shown to induce expression of various target genes that encode proteins related to different adaptive responses[38,50,58–62]. These genes contain essential HIF binding sites required for deoxyribonucleic acid (DNA) transcription activities[63–66]. The adaptive responses are induced to deliver oxygen to cells via activation of genes such as transferrin and endothelin-1 and to adapt decreased oxygen concentration through glycolysis via activation of genes such as glucose transporter 1 and lactate dehydrogenase A[5,35,67,68].

Examples of genes upregulated during hypoxia are EPO and VEGF. EPO and VEGF play critical roles in adaptive response to systemic and local hypoxia where both are activated during hypoxia[69]. VEGF plays a central role in angiogenesis, vasculogenesis and neovascularization[70,71]. Examples of transcriptional targets regulated by HIF are shown in **Figure 2.1**.

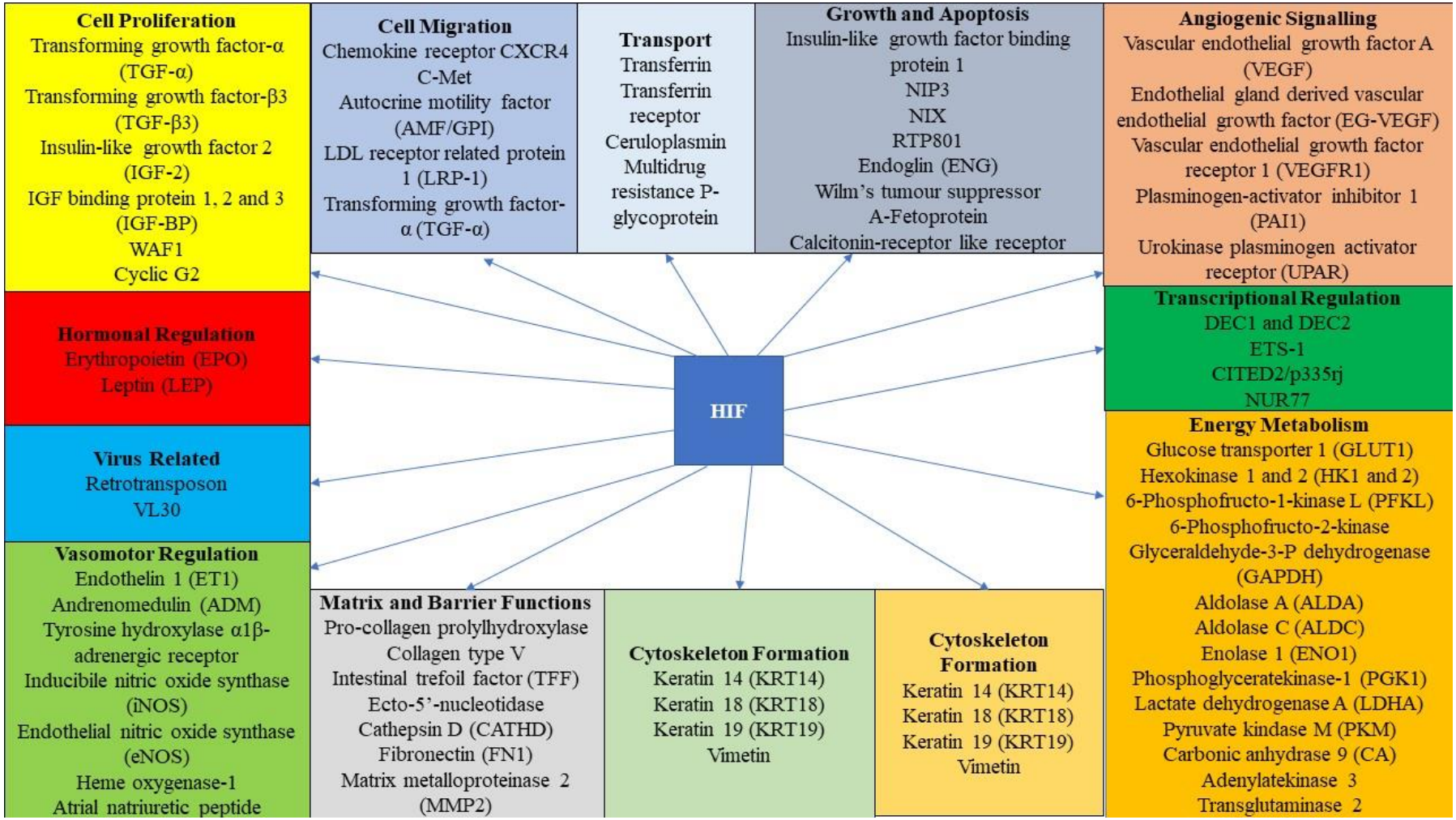


Figure 2.1: The reported transcriptional targets that are regulated by HIF. Adapted from Chowdhury, et al., 2008[72].

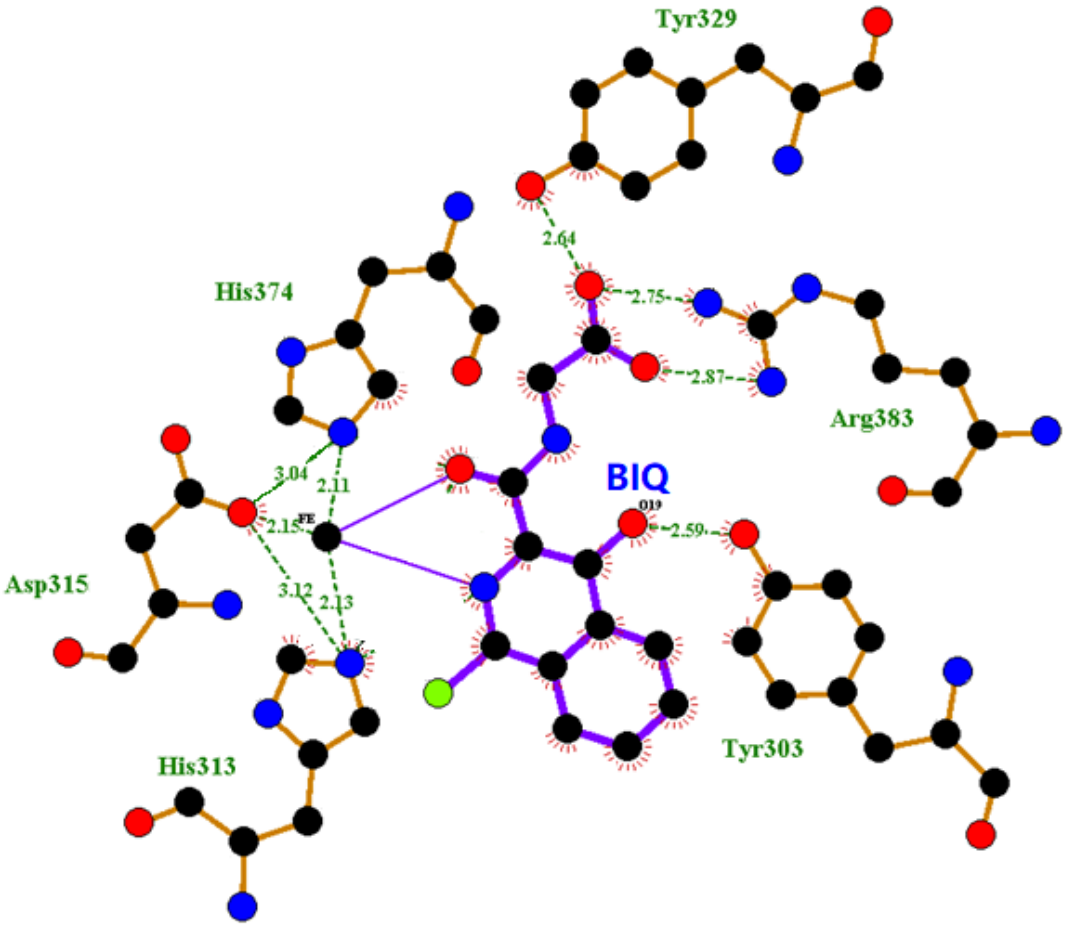
2.2 Therapeutics benefits of HIF activation

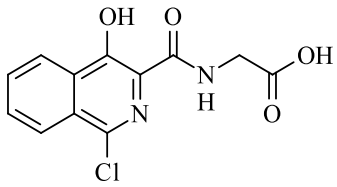
PHD inhibition by small molecule inhibitors has been shown to have a significant role in medical treatments. Various treatment strategies which promotes the stability of HIF-1α protein has been shown to play an important role in cell survival, protection against injury in cell lines and other diseases relating to inflammatory and hypoxic conditions[66,73–76]. Examples of diseases benefit from the effects of PHD inhibition are ischemia, heart attack, stroke, brain insults, chronic anemia, wounding and inflammation.

In certain cases where surgical intervention is not allowed to remove the disease, PHD inhibition has been reported to be an alternative method to be used[15,38,62,77–81]. Inhibition of PHD activity by small molecule inhibitors rather than inhibition of PHDs by hypoxia have also been shown to play a role in neuroprotection[82].

2.3 PHD-2 structural data

Up to date, out of the three PHD enzymes, PHD-2 is the only crystal structure resolved by scientist. **Figure 2.2** shows the crystal structure of 1-chloro bicyclic isoquinoline (BIQ), **1** and iron (II) ion[83]. The cofactor ferrous ion was buried deeply in the active site pocket, coordinated by three set of amino acid residues (His313, Asp315, His374) in tridentate manner forming the iron (II)/2OG complexes[83].





BIQ, 1

Figure 2.2: Binding interaction of BIQ**, 1** with PHD-2 enzyme shown in crystal structure with PDB ID: 2hbt. Data is obtained from Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB)[84].

Stabilization of the PHD-2 enzyme with inhibitor bearing aromatic hydrophobic group was due to hydrophobic nature of opening active site with presence of Trp389, Trp258, Met299 and Ile256 residues as shown in **Figure 2.3**[84]. The presence of bulky aromatic ring in PHD-2 inhibitors was an important factor as it helped to prevent HIF-1α from entering the enzyme binding pocket[54,86,87].

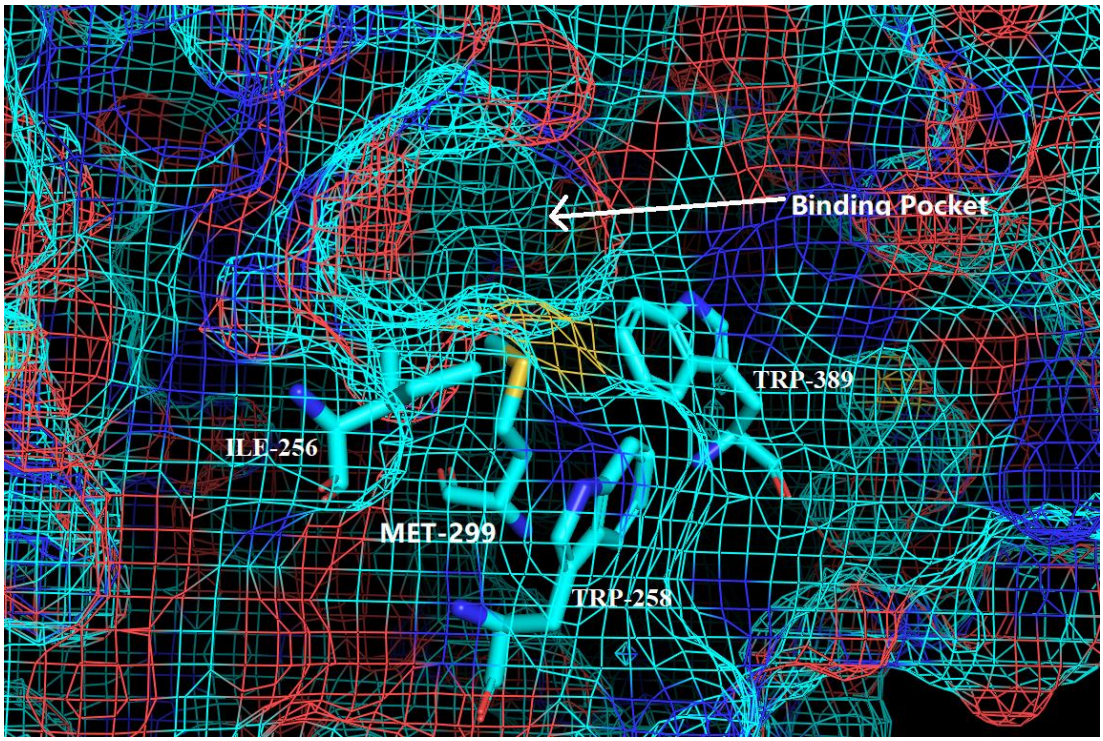


Figure 2.3: PHD-2 binding pocket with presence of Trp389, Trp258, Met299 and Ile256 residues. Data is obtained from RCSB PDB[84].

2.4 Computer aided drug design (CADD)

Computer aided drug design (CADD) was employed for the inhibitor testing in this study. Since 1960s, docking has become a powerful and essential method in drug screening, protein-ligand interactions and understanding the behavior of nanomaterials. Technologies have dominated the current field of CADD in structural-based drug design to dock ligands into receptors or proteins with many commercial drugs designed from this method[115–117].

Computational methods are preferred to be used to select potential ligands for experimental testing due to high cost and time consuming for traditional high-throughput screens. CADD method can be used to estimate the binding affinity and binding mode of ligand to the drug target in short period of time. CADD has important role in therapeutic applications due to the improvement of structural studies on

biological molecules which helps in computational approaches for small molecule docking and virtual screening of candidate compounds. Potent and selective inhibitors are designed by medicinal chemists utilizing three-dimensional structures of various protein targets[115–117].

Structural information of binding site obtained from X-ray crystal structures are used in protein-ligand docking methods[118]. The increase in numbers of high-resolution crystal structure of receptors in recent years bring to an increase in computation studies of molecular docking. Potential ligands can be identified once structural information about macromolecular drug target is known. There is a growing interested in pharmaceutical industry due to increase in accuracy. Binding affinities of selected ligands are predicted via associating scoring functions with potent ligand chosen from the best binding affinity to run further biochemistry experiments and development[119–121].

Drug or inhibitor design process determines the binding and orientation of a ligand in a protein receptor complex which utilizes computational technologies. Computational technologies enable rapid identification of hit and lead compounds. Binding mode and affinity are important in designing therapeutic interventions. Binding mode is the pose with lowest energy score predicted as the best match. Binding affinity is the strength of the binding interaction between ligands and receptor[119,121]. Knowledge of biological target of interest is used to optimize the process of finding new modifications.

Identification of biological target of interest is the early step in virtual screening[122]. Virtual screening utilizes docking as routine in drug screening and design and performs docking for large number of compounds to determine the putative leads with

given modelled structure of receptor[120,123,124]. Compounds search via this method is pharmaceutically interesting at a higher rate with a lower cost.

There are two types of virtual screening: ligand-based virtual screening and structure-based virtual screening. Properties of set of ligands known to bind to the receptor in interest are important for a ligand's biological effects in ligand-based virtual screening. For structure-based virtual screening, ligands are the physical entities and scoring function is used to predict the binding site of interest.

After structures of ligands and proteins are prepared, energy minimization is utilized to determine the most stable 3D confirmation. Ligands are drawn and protein to be docked can be downloaded from PDB with focus on higher X-ray resolution for better accuracy.

2.4.1 Docking and root mean square deviation (RMSD)

Docking is a very important technique for understanding interaction of chemical compounds with enzymes. It is also a simple technique because it requires very low equipment to run. Docking begins with the idea of 'lock and key' drug design. Protein-ligand docking usually involves sampling algorithms and scoring functions. Sampling algorithms in docking software is used to explore the possible binding modes of ligand in the protein-ligand complex while scoring function is used to estimate relative binding affinities and rank ligand poses[121,125–127].

Docking focuses on creating the correct conformation of the designed compound in the receptor with the cocrystalline compound. Root mean square deviation (RMSD) value refers to the ability of the docking programs to replicate the binding mode of

reported compound with the cocrystalline compound in the target protein. Values threshold of RMSD below 2 Å between reported and cocrystalline compounds are statistically the most frequently used to predict the success rate while RMSD below 1.5 Å was preferred for smaller compounds[128–132]. In this study, PyMOL 2.0.7 by Schrödinger, LLC was used to determine the RMSD of the designed compounds.

2.5 PHD inhibitors

The hydroxylation of HIF by PHDs requires oxygen, 2OG and iron (II) ion. There are a few classes of PHDs reported inhibitors, including iron chelators, transition metal ions and mimetic 2OG inhibitors. The carboxylate group in mimetic 2OG inhibitors was shown to compete with 2OG in the enzyme binding pocket in certain studies[54]. The design of carboxylate appendage inhibitors was focused in study.

2.5.1 Iron chelators

Iron chelators inhibit PHDs by chelating the iron (II) ion active site, hence, the enzymes lose its function to hydroxylate HIF. Three reported PHD inhibitors which work via chelation of iron are desferrioxamine (DFO), **2**, clioquinol**, 3** and ciclopirox, **4**. Their structures are shown in **Figure 2.4**. These iron chelators are able to upregulate HIF-1α levels, but failed to upregulate erythropoietin plasma[38,40,88]. Clioquinol and ciclopirox are low-affinity inhibitors which are not selective for PHD-2 enzyme[88].

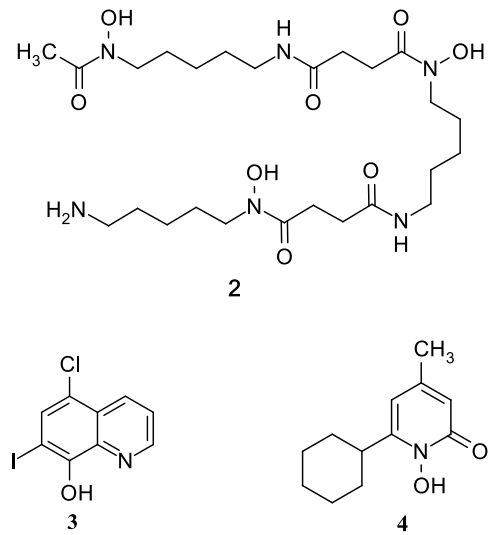


Figure 2.4: Structures of different iron chelators: **2**, **3** and **4**.

2.5.2 Transition metal ions

Transition metal ions inhibit PHDs by displacing the iron (II) ion active site. Examples of transition metal ions inhibitors are Co^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+} and Mn^{2+} [89,90]. The half-maximal inhibitory concentration (IC_{50}) against PHD-2 by transition metal ions are summarized in **Table 2.1**.

Table 2.1:Reported IC_{50} values of the transition metal ions against PHD-2[90].

Metal ions	**Co^{2+}**	**Ni^{2+}**	**Zn^{2+}**	**Cu^{2+}**	**Cd^{2+}**	**Mn^{2+}**
IC_{50} (μM)	48.3	185.2	9.3	6.6	57.4	21.0

2.5.3 2-Oxoglutarate (2OG) mimetic inhibitors

2OG mimetic inhibitors are compounds that have similar structures as 2OG, **5**. They can compete with co-substrate 2OG to chelate the metal ion and form hydrogen bonding in the active site of PHD-2. Examples of 2OG mimetic inhibitors are N-oxalylglycine (NOG)[91], thiazol analogues[92], pyrazole analogues[34,93–95], pyridine

analogues[34,93,96,97], pyrimidine[98], benzodiazole analogues[94,99] and bicyclic isoquinoline analogues[97,100–102].

2OG mimetic inhibitors have similar binding interaction in the PHD-2 active site as the co-substrate 2OG. The crystal structure of **2OG** in PHD-2 is shown in **Figure 2.5**. Bidentate coordination with the iron (II) ion active site are formed from the oxygen atoms while salt bridge with Arg383 residue and hydrogen bonding with Tyr329 residue are formed from oxygen atoms in the carboxylate group[94].

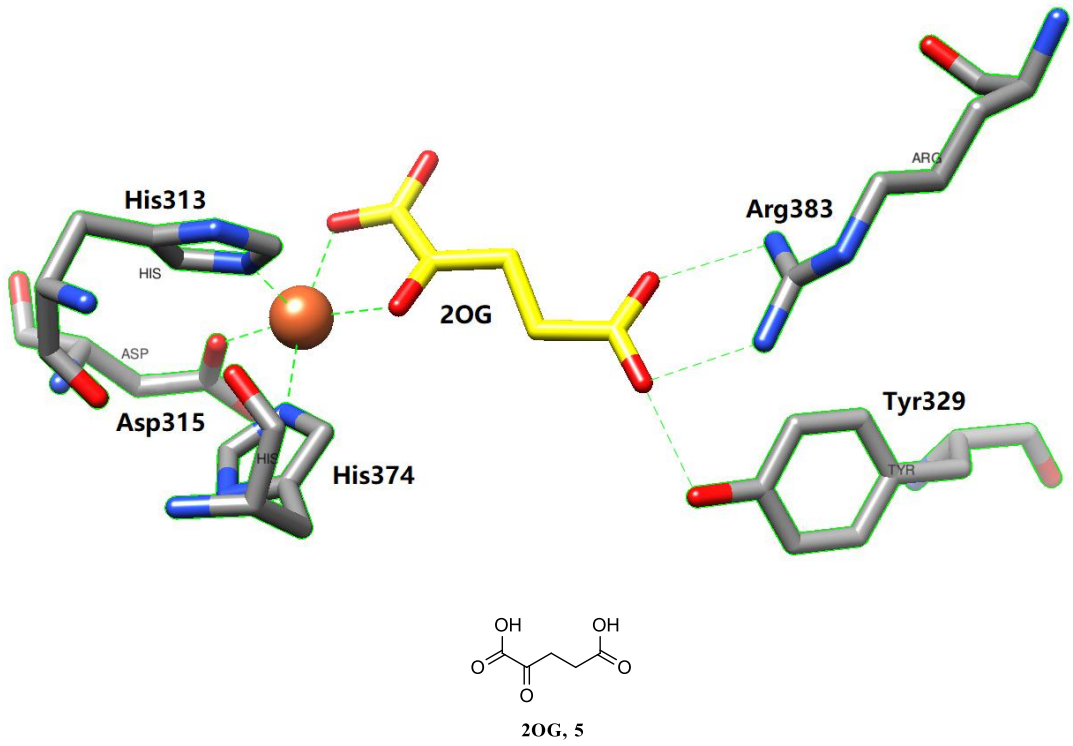


Figure 2.5: The crystal structure of PHD-2 active site with **2OG**. **2OG** is shown in yellow while iron (II) is shown in brown[94]. Data is obtained from RCSB PDB[84].

2.5.3(a) N-oxalylglycine (NOG) and dimethyl N-oxalyl-glycine (DMOG)

NOG, **6** has been shown to inhibit PHDs in competition with **2OG**. An ester derivative form of **NOG**, DMOG, **7** is a cell permeable prodrug of **NOG** which penetrates the cells to form **NOG**. The process is reported to be catalyzed by cellular

carboxylesterases[13,103]. **DMOG** is shown to inhibit activity of both PHDs and FIH and upregulate HIF-1 and HIF activity in animals[80,104]. IC_{50} value of **NOG** against PHD-2 is shown in **Figure 2.7**. The binding interaction of **NOG** is shown in **Figure 2.6**.

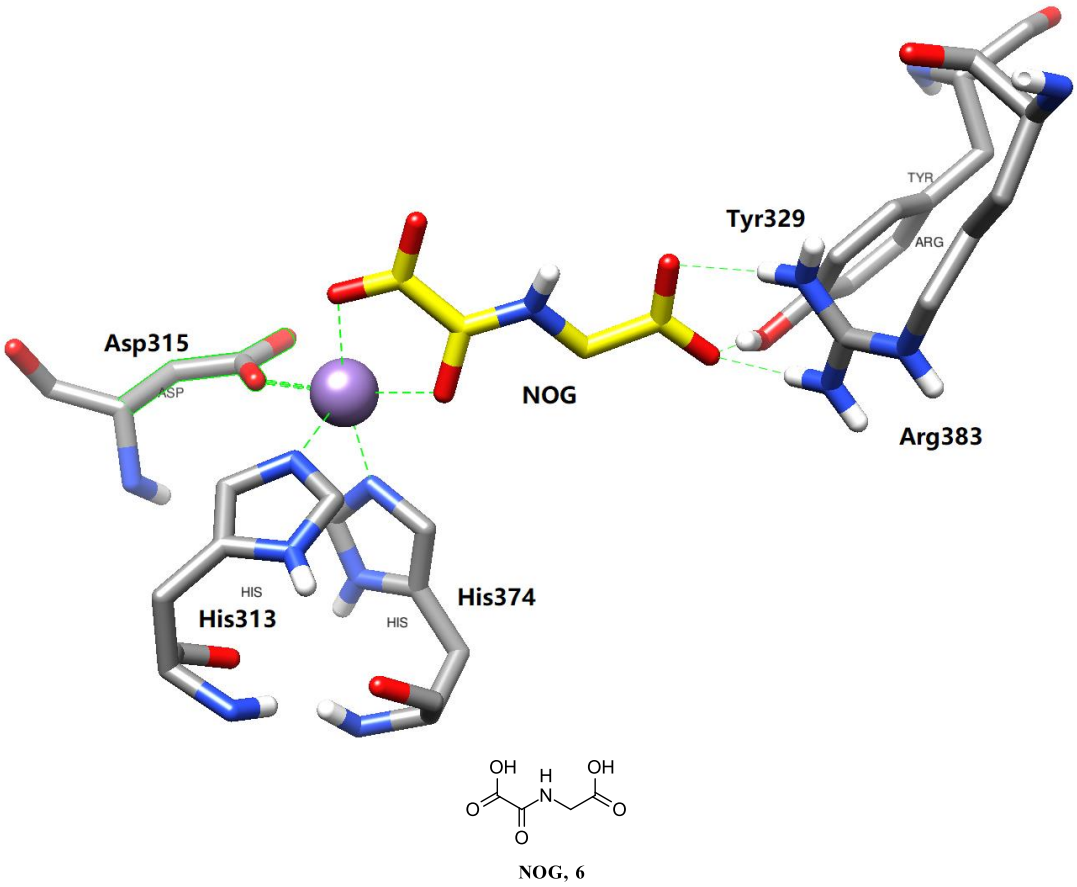
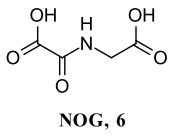
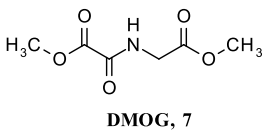


Figure 2.6: The crystal structure of PHD-2 active site with **NOG, 6**. **NOG, 6** was shown in yellow while manganese (II) was shown in purple was used instead of iron (II)[91]. Data is obtained from RCSB PDB[84].



NOG, 6



DMOG, 7

Figure 2.7: **NOG, 6** and **DMOG, 7** with reported IC_{50} values of **NOG** (6.2[105] **μM**) against PHD-2.

2.5.3(b) Thiazol and pyrazole analogues

A series of thiazol and pyrazole analogues were reported as PHD-2 inhibitors. The thiazol compound **8** with a benzene ring further away from the thiazol group and carboxylate side chain closer to the thiazol group was shown to have better inhibition potency compared to compound **9.** On the other hand, the pyrazol compounds **12** and **14** with a bicyclic ring structure seems to exhibit better inhibition potency compared to compounds (**11, 13** and **15**) with monocyclic ring structure. The position of the benzene ring of compounds (**8**, **9** and **10**) further away from the thiazol group might exhibit better inhibition potency due to better fitting in the narrow active site of PHD-2 enzyme. Bicyclic ring structures (**12**, **14** and **16**) showed to have better inhibition value in pyrazole analogues might be due to the resonance of the electrons in the ring structure which favors the coordination bond with the iron (II) ion. The structures of thiazol (**8 - 10**) and pyrazole analogues (**11 - 16**) were shown in **Figure 2.8** and their IC_{50} values against PHD-2 were summarized in **Table 2.2**. An example of crystal structure of pyrazol compound **16** in PHD-2 active site is shown in **Figure 2.9**. Bidentate coordination with the active site iron (II) ion are formed from the nitrogen atom in benzimidazole and pyrazole while salt bridge with Arg383 residue and hydrogen bonding with Tyr329 residue are formed from oxygen atom in carboxylate group.

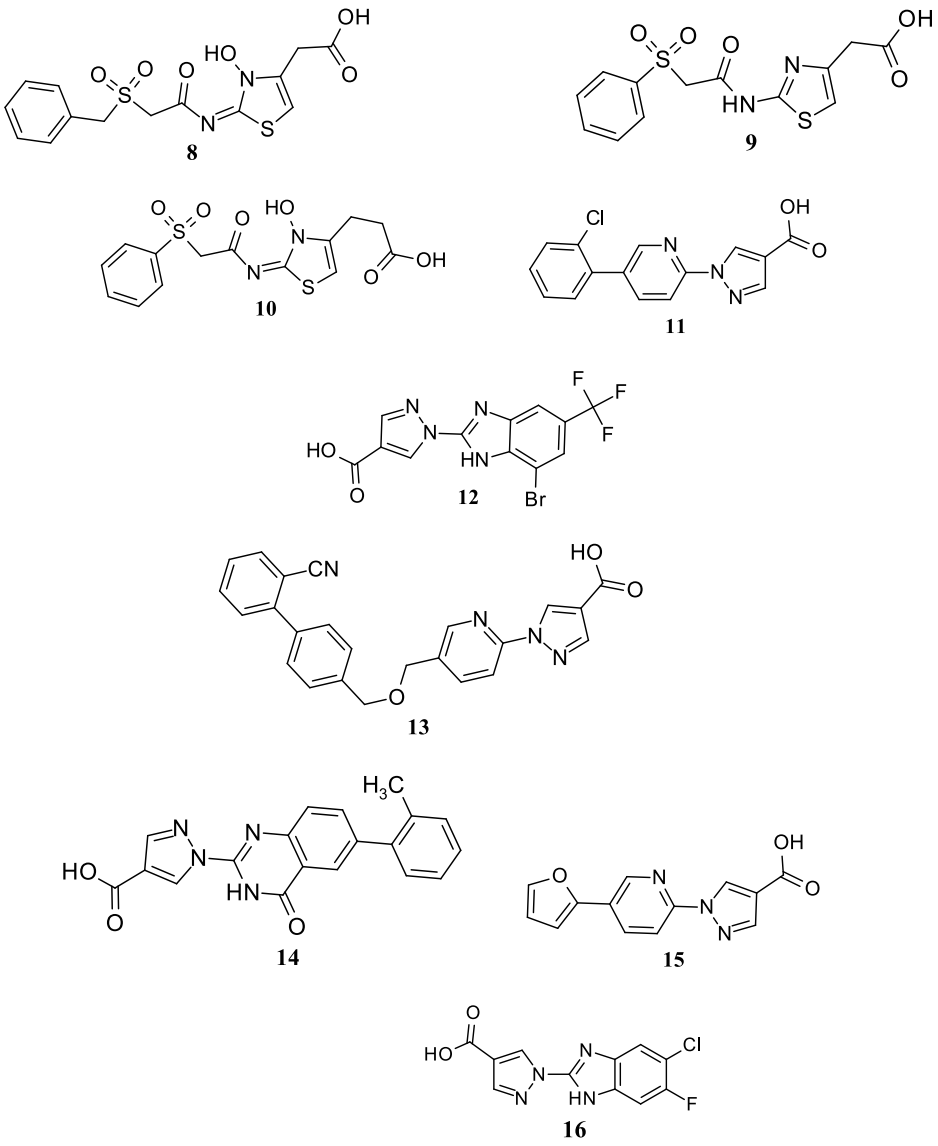
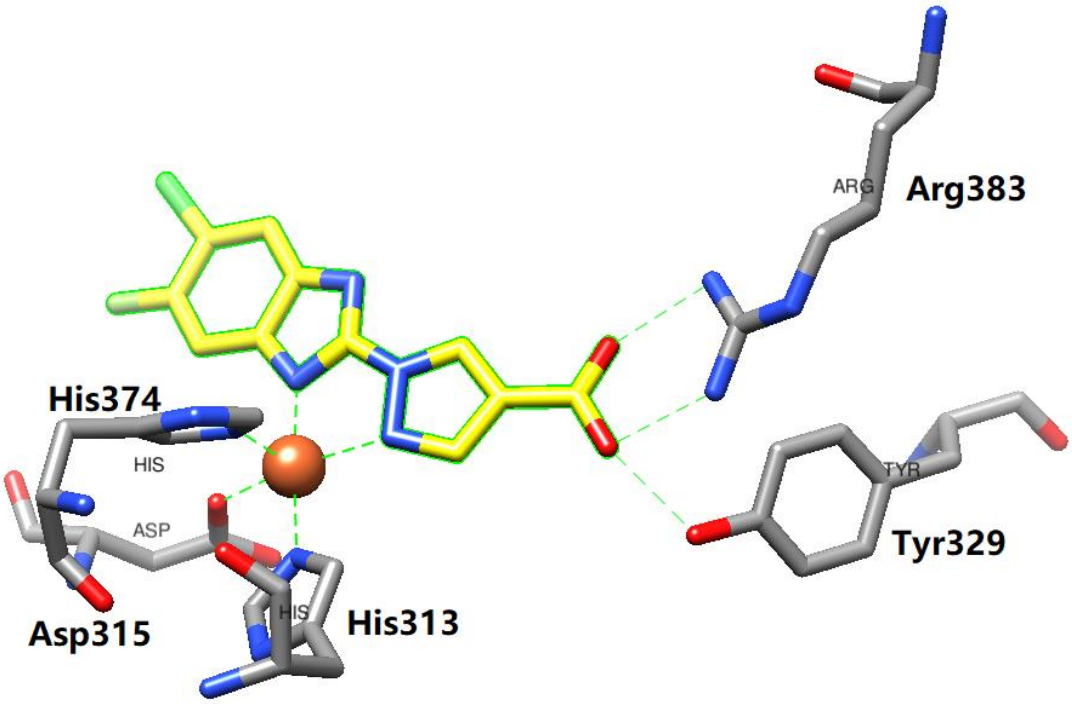
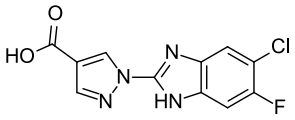


Figure 2.8: Thiazol analogues (**8 – 10**) and pyrazole analogues (**11 – 16**).

Table 2.2: Reported IC_{50} values of thiazol analogues (**8 – 10**) and pyrazole analogues (**11 – 16**) against PHD-2.

Compound	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
IC_{50} (µM)	0.011[92]	40[92]	0.41[92]	6.5[93]	0.1585[94]	0.19[95]	0.008[34]	15[93]	0.07943[94]





16

Figure 2.9: The crystal structure of PHD-2 active site with 1-(5-chloro-6-fluoro-1H-benzimidazol-2-yl)-1H-pyrazole-4-carboxylic acid, **16**. **16** was shown in yellow while iron (II) was shown in brown[94]. Data is obtained from RCSB PDB[84].

2.5.3(c) Pyridine analogues, pyridinol analogues and pyrimidine

A series of reported pyridine analogues, pyridinol analogues and pyrimidine potent PHD-2 inhibitors are shown in **Figure 2.10**. Compound **18** with additional monocyclic ring away from the carboxylic group with the purpose of blocking the entrance of the PHD-2 active site does not give better inhibition value compared to compound **17** which does not have any benzene ring structure that blocks the entrance of the PHD-2 active site. This might be due to the absence of the hydroxyl group in the aromatic ring because compounds **19** – **23** have hydroxyl group in the aromatic ring adjacent to the carboxylic group. The crystal structure of compounds **22** and **23** were shown in **Figure**

2.11 and **Figure 2.12,** respectively. The IC_{50} values of pyridine analogues, pyridinol analogues and pyrimidine were shown in **Table 2.3**.

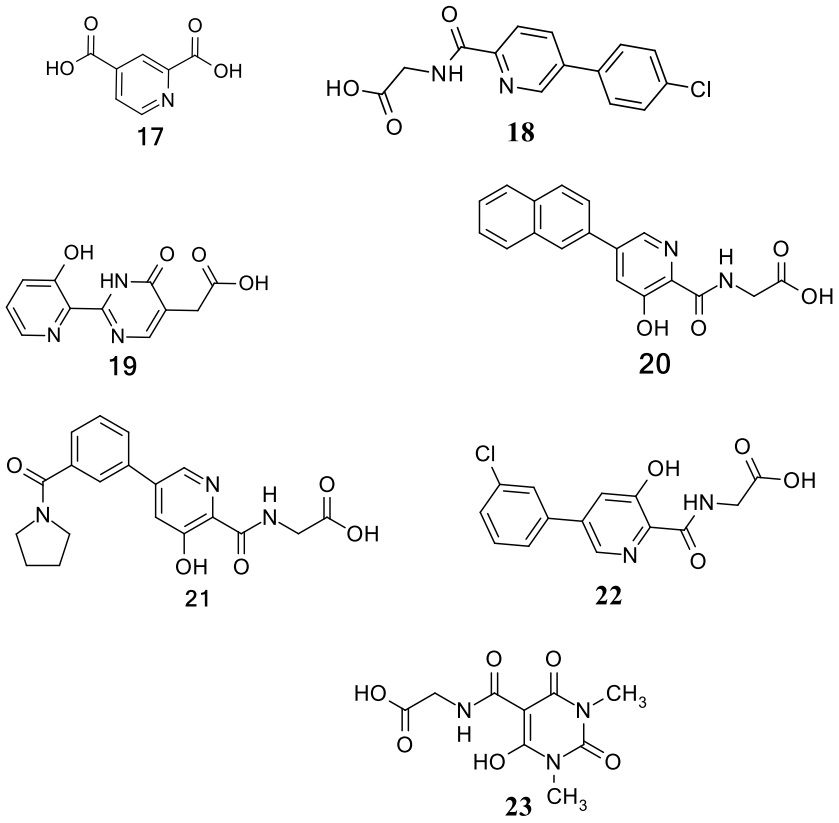
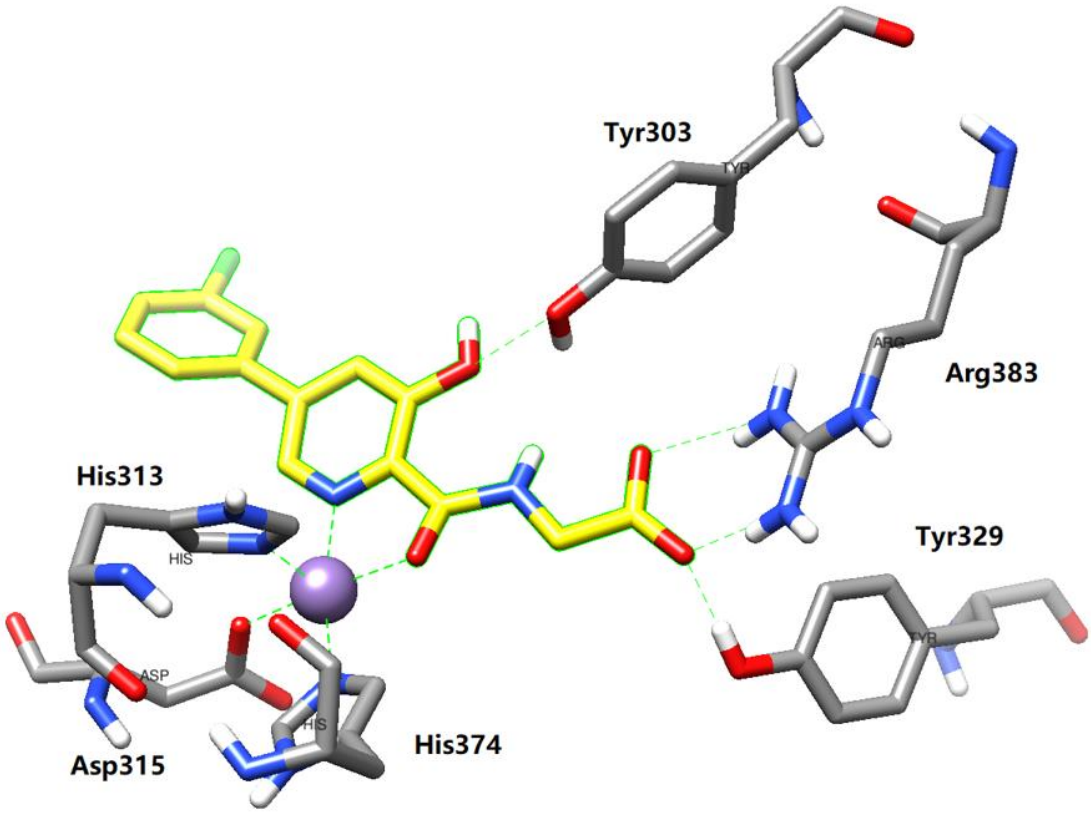
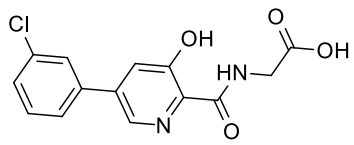


Figure 2.10: Pyridine analogues (**17 - 18**), pyridinol analogues (**19 - 22**) and pyrimidine (**23**).

Table 2.3: Reported IC_{50} values of pyridine analogues (**17 – 18**), pyridinol analogues (**19 - 22**) and pyrimidine (**23**) against PHD-2.

Compound	**17**	**18**	**19**	**20**	**21**	**22**	**23**
IC_{50} (μM)	2[97]	15[93]	27.5[106]	0.017[34]	0.3[107]	0.029[98]	0.067[98]





22

Figure 2.11: The crystal structure of PHD-2 active site with Vadadustat, **22**. **22** was shown in yellow while manganese (II) (substituting iron (II)) was shown in purple[98]. Data is obtained from RCSB PDB[84].

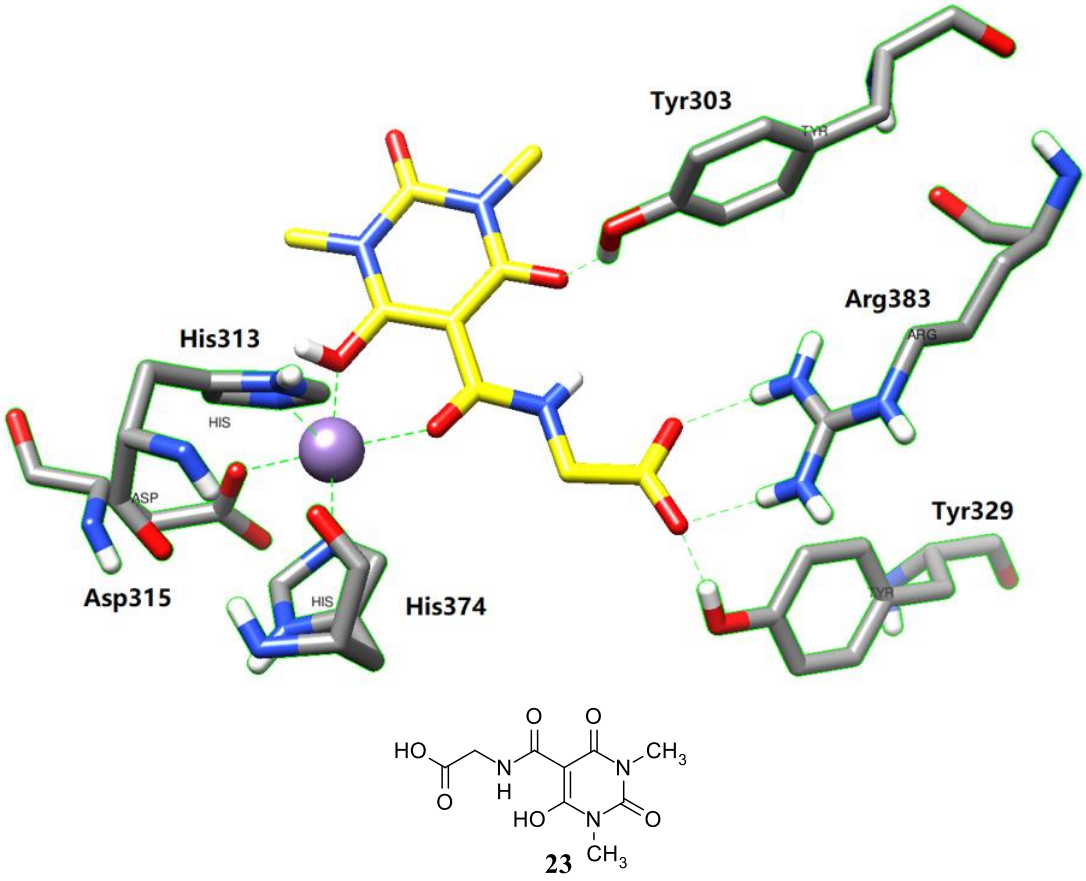


Figure 2.12: The crystal structure of PHD-2 active site with (6-hydroxy-1,3-dimethyl-2,4-dioxo-1,2,3,4-tetrahydropyrimidine-5-carbonyl)glycine, **23**. **23** was shown in yellow while Mn(II) (substituting Fe(II)) was shown in purple[98]. Data is obtained from RCSB PDB[84].

2.5.3(d) Benzodiazole analogues

Examples of benzodiazole analogues that were reported as potent PHD-2 inhibitors were shown in **Figure 2.13**. The inhibition potency is higher when there is a benzene ring attached to the bicyclic ring structure as shown in compound **25**. The crystal structure of benzodiazole analogues were shown in **Figure 2.14** and the IC_{50} values were shown in **Figure 2.13**. Bidentate coordination with the iron (II) ion active site are formed from the oxygen atom in amide group and nitrogen atom in benzimidazole group while salt bridge with Arg383 residue and hydrogen bonding with Tyr329 residue are formed from oxygen atom in carboxylate group as shown in **Figure 2.14**[94].