Bioorganic & Medicinal Chemistry 21 (2013) 6349–6358

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Novel complex crystal structure of prolyl hydroxylase domain-containing protein 2 (PHD2): 2,8-Diazaspiro[4.5]decan-1-ones as potent, orally bioavailable PHD2 inhibitors

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ARTICLE INFO

Article history:
Received 8 August 2013
Revised 24 August 2013
Accepted 24 August 2013
Available online 3 September 2013

Keywords:
2,8-Diazaspiro[4.5]decan-1-one
Prolyl hydroxylase domain-containing protein 2 (PHD2)
PHD inhibitors
Hypoxia inducible factor (HIF)
PHD2 crystal structure
SAR study

ABSTRACT

We have discovered a novel complex crystal structure of the PHD2 enzyme with its inhibitor, the 2,8-diazaspiro[4.5]decan-1-one analogue **4b**. The widely reported salt bridge between Arg383 of the enzyme and its inhibitors in all complex structures published thus far was not observed in our case. In our complex structure compound **4b** forms several novel interactions with the enzyme, which include a hydrogen bond with Arg322, a π-cation interaction with Arg322, a π–π stacking with Trp389, and a π–π stacking with His313. Guided by the structural information, SAR studies were performed on the 2,8-diazaspiro[4.5]decan-1-one series leading to the discovery of compound **9p** with high potency and good oral pharmacokinetic profile in mice.

1. Introduction

Prolyl hydroxylase domain-containing protein 2 (PHD2), also known as egg-laying defective nine homologue 1 (EGLN1) or HIF prolyl hydroxylase 2 (HPH2), is one of three known 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenases (PHD1-3).[1] Primarily localized in cytoplasm, PHD2 is highly expressed in heart and testis while moderately expressed in brain, kidney, and liver.[2] PHD2 plays an important role in cell adaptation and survival, responding to the oxygen environment, by regulating the hypoxia-inducible transcription factor (HIF).[3,4] Under normoxia, hypoxia inducible factor 1α (HIF1α), an α-subunit of HIF, was reported to be rapidly degraded after oxidation by PHD enzymes, which were identified to use O_2 and 2OG as co-substrates to generate 4-hydroxyprolines at residues Pro402 and/or Pro564 of HIF1α.[5–8] Once PHD1-3 activities are inhibited, as they would be under hypoxia conditions or by small molecules competing for 2OG or Fe(II) binding, the oxidative degradation of HIFα subunits would be impeded resulting in the up-regulation of HIF, which in turn leads to the up-regulation of erythropoietin (EPO), an essential growth factor for the production of red blood cells (RBCs).[9–13] Thus, inhibition of PHD enzymes has emerged as an attractive strategy for the treatment of anemia.[14]

In this work, we have focused on PHD2, the most extensively studied PHD enzymes and also the key oxygen sensor keeping low steady-state levels of HIF1α in vivo under normoxia.[5,14] Quantities of PHD2 enzyme were isolated and purified according to the literature report[15] for the high-throughput screening (HTS) of an internal compound library. Spiro compounds **1** and **2** (Fig. 1) with moderate/low inhibition potencies (pIC_{50} 6.0 and 4.9, respectively) in the PHD2 HTRF (homogeneous time resolved fluorescence) assay were identified from the HTS. By searching the literature, we found that similar structures (e.g., compound **3**) have been reported[11,16] and the compound (**3**) demonstrated potent enzymatic inhibition (pIC_{50} 9.3). As a continuous effort to improve compounds' physicochemical properties such as reduced number of aromatic rings and increased 3-dementionality,[17] we decided to remove the phenyl ring fused to the central spiro core of compound **3** for simplified structures such as the 2,8-diazaspiro[4.5]decan-1-one derivatives **4a** and **4b**. To our delight, the new structure series showed reasonable PHD2 inhibitory potency (pIC_{50} 7.6 and 7.8 for **4a** and **4b**, respectively) thus served as our chemistry starting point. Herein, we will report the novel complex crystal structure of PHD2 enzyme with **4b** and the SAR studies to discover the 2,8-diazaspiro

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http://dx.doi.org/10.1016/j.bmc.2013.08.046

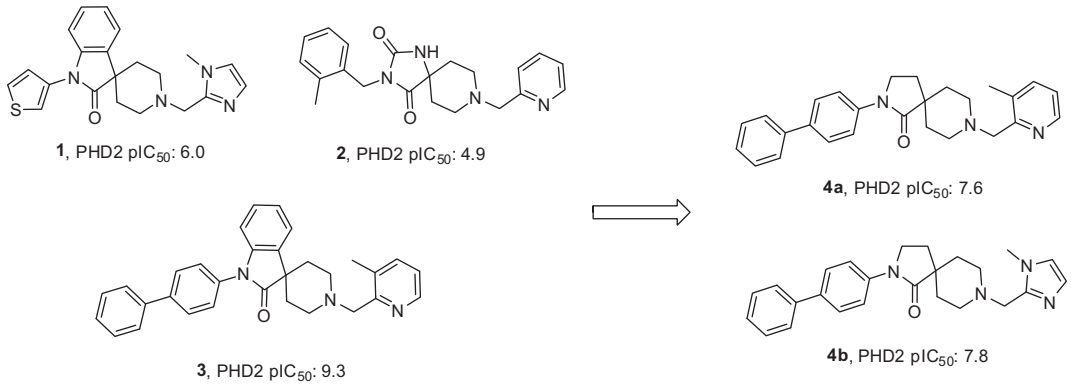


Figure 1. HTS hits **1** and **2**, literature compound **3** and newly designed compounds **4a** and **4b**.

[4.5]decan-1-one derivatives as potent, orally bioavailable PHD2 inhibitors.

2. Results and discussion

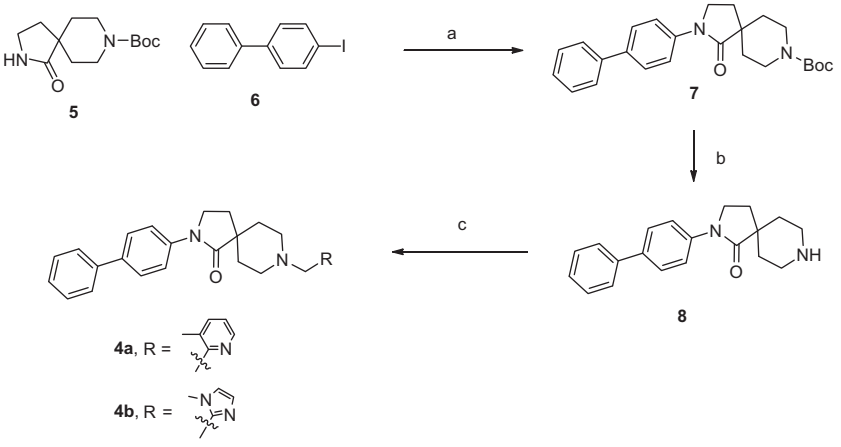
2.1. Chemistry

The synthetic route for compounds **4a** and **4b** (and their close analogues) was presented in Scheme 1. Copper(I)-catalyzed Ullmann coupling of commercial starting materials spiro lactam **5** and biphenyl iodide **6** provided intermediate **7**. The *tert*-butoxycarbonyl (Boc) protective group was removed under the acidic condition to afford amine **8**. Reductive amination reactions of **8** with 3-methylpicolinaldehyde or 1-methyl-1*H*-imidazole-2-carbaldehyde provided the desired products **4a** and **4b**, respectively. Compounds **4c–l**, **9a–s** were prepared under similar protocol. All compounds (**4a–l**, **9a–s**) were purified by mass directed auto-purification system (HPLC purity >95%) and their spectroscopic data (HRMS, ^{1}H NMR, and/or ^{13}C NMR) were consistent with the structure assignments.

2.2. Crystallography

We firstly undertook structural studies to elucidate the mode of inhibition of the series to guide SAR studies. Fortunately, we were able to obtain the X-ray crystal structure of a truncated PHD2 enzyme (residues 181–418) complexed with compound **4b** (Fig. 2) by soaking. In the complex structure, PHD2 has a double-stranded β-helix fold common for 2OG-dependent dioxygenases, comprising eight β-strands packed around by three α-helices similar to previous reports.[15] There is a single Ni(II), in place of Fe(II), coordinated in an octahedral manner by His313, Asp315, His374, compound **4b**, and a water molecule. Compound **4b** chelates with Ni(II) through the 3-N atom of the imidazole (1.9 Å from the metal) and the tertiary amine of the piperidine (2.3 Å), and this metal chelation interaction is an important binding interaction between the PHD2 enzyme and its inhibitors.[12,15]

Besides metal chelation, the salt bridge interaction in the active site between Arg383 of the PHD2 enzyme and the carboxylic acid functional group of its inhibitors/natural ligand has been consistently observed in all reported complex structures (Fig. 3), and this interaction is considered to be critical for achieving potent inhibition activity.[12,15,18] In our complex structure of PHD2 enzyme and compound **4b**, the salt bridge was not observed. Instead, we observed several novel interactions. Compound **4b** forms a strong hydrogen bond (donor–acceptor distance at 3.0 Å, donor-hydrogen-acceptor angle at 162°) with Arg322 through its carbonyl group on the spiro core. Arg322 also forms a cation–π interaction (distances between 3.3 and 3.9 Å) with the phenyl ring directly attached to the spiro core. In addition, a π–π stacking (edge-to-face, hydrogen–carbon distance at 2.6 Å, two rings are perpendicular) between the same phenyl ring of **4b** with Trp389 of PHD2 was observed. Furthermore, a π–π stacking (face-to-edge, nitrogen–hydrogen distance at 2.6 Å, two rings are nearly perpendicular)



Scheme 1. General synthetic procedure for the 2,8-diazaspiro[4.5]decan-1-ones **4a**, **4b**, and analogues. Reagents and conditions: (a) CuI, K_2CO_3, *N1*,*N2*-dimethylethane-1,2-diamine, Acetonitrile, 80 °C, 36%; (b) TFA, DCM, 23 °C, 5 h, 100%; (c) sodium triacetoxyborohydride, 3-methylpicolinaldehyde or 1-methyl-1*H*-imidazole-2-carbaldehyde, 2 h, 26% for **4a** and 41% for **4b**.

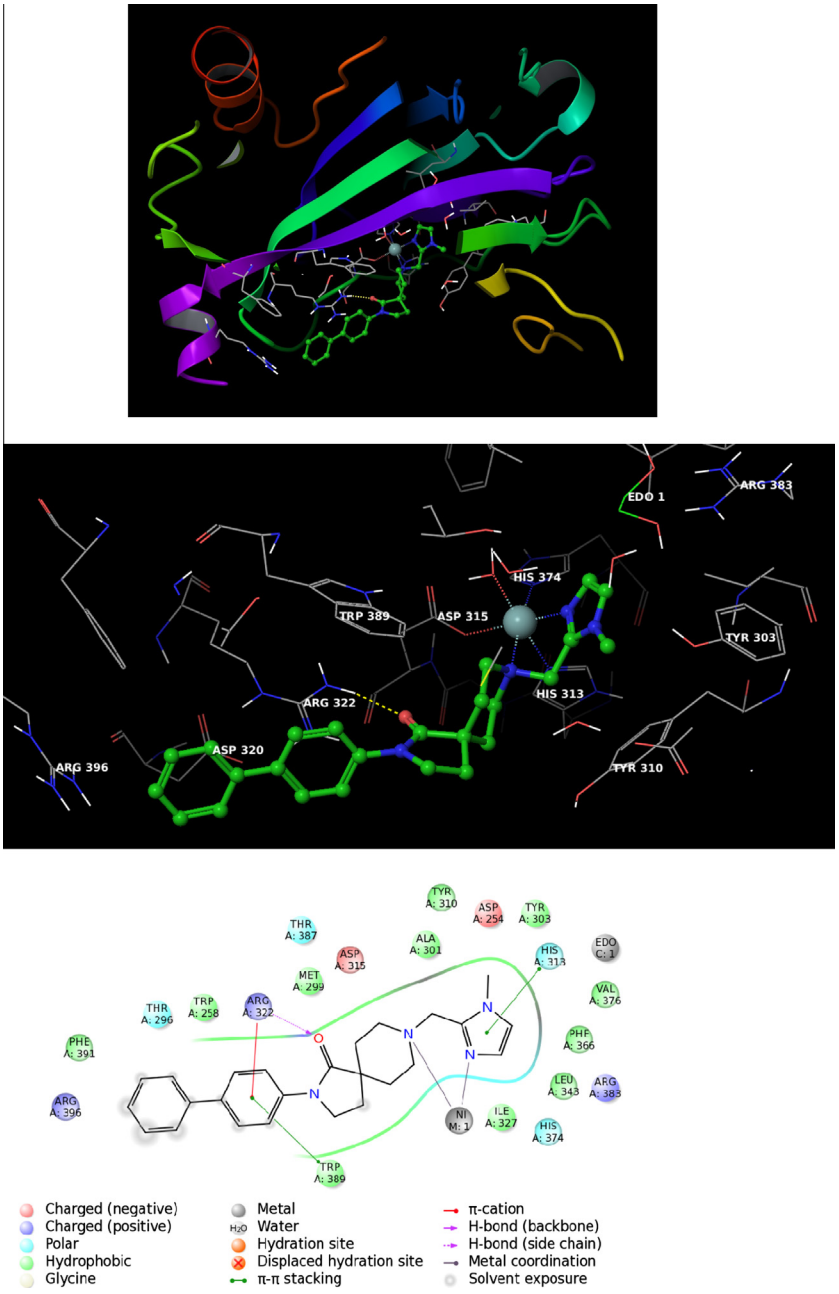


Figure 2. Crystal structure of truncated PHD2 enzyme complexed with compound **4b** (PDB: 4JZR). The structure and interaction diagram are drawn in Maestro [Schrödinger 2012R1].

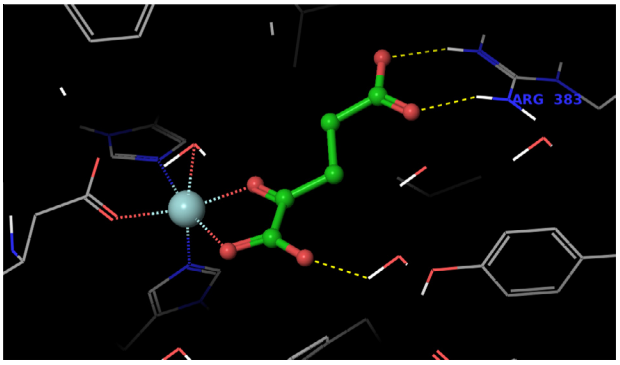


Figure 3. Crystal structure of PHD2 with co-substrate 2OG (PDB: 3OUJ[12]).

of the imidazole moiety of **4b** with His313 of PHD2 was also identified from the crystal structure. These π–π stacking interactions are usually weaker than hydrogen bonds or salt bridges. The biphenyl moiety of **4b** is exposed to solvent, and not much hydrophobic interaction can be identified. Compound **4b** clearly does not form salt bridges with Arg383 (more than 5 Å away in distance, Fig. 2) which, besides the chelation interaction, was reported to be critical for the potency of PHD2 inhibitors (Fig. 3).[12,15,18] Without this interaction, it is thus intriguing how compound **4b** can achieve such a high potency (pIC_{50}: 7.8). To understand this, we studied the crystal structure of PHD2 complexed with its substrate HIF-1α to see whether compound **4b** can interfere with their binding (Fig. 4). HIF1α has several key interactions with PHD2, including but not limited to the salt bridge between Asp571 of HIF1α and Arg396 of PHD2 and also the hydrogen bonds between Pro564

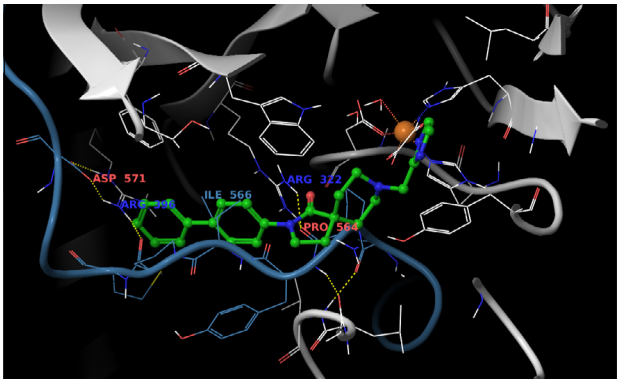


Figure 4. Crystal structure of PHD2 with substrate HIF1α [PDB: 3HQR[19]] in overlay with compound **4b**. PHD2 is shown in white, HIF1α peptide in blue, and compound **4b** in green.

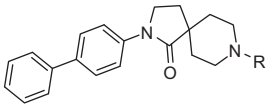
(HIF1α hydroxylation site) and Arg322 of PHD2. By overlaying this crystal structure with that of PHD2 complexed with compound **4b**, one can clearly see that compound **4b** overlaps with both of these two interactions as well as with Ile566 of HIF1α. This indicates that, although compound **4b** might not compete for the co-substrate binding site (2OG) as strongly, it may compete extensively for the substrate (HIF1α) binding site. We speculate that it is the combination of these two properties that confer high potency to this compound.

2.3. In vitro pharmacology

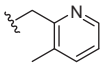
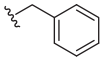
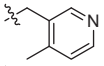
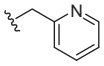
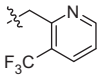
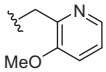
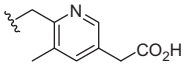
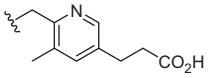
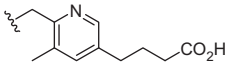
2.3.1. SAR study on the right-hand side pyridine substitutions

It was challenging to get the complex structure of PHD2 enzyme with compound **4a**. Given the structural similarity, we proposed that the pyridine nitrogen of **4a** formed a metal chelation interaction with PHD2 which is critical for potency. This hypothesis was supported by the loss of potency when the pyridine nitrogen was replaced with carbon (**4c**, Table 1). In addition, moving the nitrogen atom away from the chelation position of the pyridine also provided inactive analogues (**4d** and **4e**). The 3-methyl substitution on the pyridine proved to be important for potency as well and the compound without this methyl substitution (**4f**) demonstrated ~10-fold decrease in potency. Replacements of the 3-methyl group with electron withdrawing (chloro, trifluoromethyl) and electron donating (methoxyl) groups (**4g**, **4h**, and **4i**) resulted in 10- to 15-fold decreases in potency, which might be due to reduced ability of the pyridine to form chelation interactions with Fe(II) or decreased π–π stacking (edge-to-face) interaction with His313. To further improve potency, compounds **4j**, **4k**, and **4l** with carboxylic acid side chains were designed to form salt bridge interactions with Arg383 of the PHD2 enzyme which have been identified as one of the key interactions responsible for high potencies of 2OG mimics.[12,15,18] As expected, the carboxylic acid analogues (**4j** and **4k**) demonstrated more than 10-fold increase in potency compared with **4a**, indicating the formation of the salt bridge. This interaction was further supported by docking compound **4k** with the enzyme (Fig. 5). Compound **4l**, however, showed much decreased potency (~100-fold) compared with **4i** and **4k** likely due to the longer carboxylic side chain making the salt bridge interaction less optimal. Although the carboxylic acid analogue (**4k**) demonstrated sub-nanomolar potency, it is not a developable compound due to its poor PK profile. Very low peripheral exposure ($DNAUC_{0\sim t}$: 22.6 (ng h/mL)/(mg/kg)) and high hepatic extraction (E_H = 0.68) were observed for compound **4k** in the mouse short oral absorption (SOA) study, a mouse in vivo oral PK

Table 1
SAR on the right-hand side pyridine substitutions (**4**)



4

Compound	R	PHD2 pIC_{50}[a]
4a		7.6
4b		7.8
4c		<5
4d		<5
4e		<5
4f		6.8
4g		6.7
4h		6.1
4i		6.2
4j		8.6
4k		9.0
4l		7.1

[a] PHD2 assay data is the average of at least two determinations.

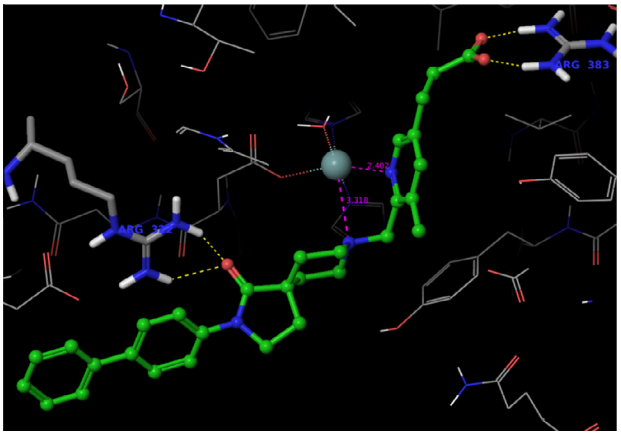


Figure 5. Docking study of the carboxylic acid analogue **4k** indicating salt bridge interactions with the PHD2 enzyme. Docking was performed using Glide [Schrödinger 2012R1].

study. We then focused our efforts on the left-hand side biphenyl moiety to improve the series' oral exposure while maintaining potency.

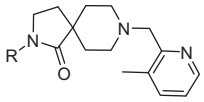
2.3.2. SAR study on the left-hand side bi-phenyl moiety

Our SAR efforts were focused on reducing series' lipophilicity (*c*Log*P*) for better ADMET profile.[19] As shown in Table 2, a variety of analogues with reduced *c*Log*P* were prepared and evaluated. When the terminal phenyl group was replaced with a more polar and smaller ethoxyl group (**9a**), the potency was decreased by ~100-fold. Increasing the size of the ethyl group to a sterically bulkier isopropyl group (**9b**) provided only negligible increase in potency compared to **9a**. The oxygen atom of **9a** was then removed to explore the hydrophobic effect at this position, and the resultant compound **9c** showed marginal activity. Replacement of the terminal phenyl group of **4a** with a saturated cyclohexyl ring (**9d**) resulted in ~80-fold decrease in potency. The more polar morpholine analogue (**9e**) was almost inactive. These SAR data suggested that the aromatic ring was preferred at the terminal position. We then designed different six-membered heteroaromatic rings such as pyridine (**9f**) and pyrimidine (**9g**) but both compounds proved to be barely active. In addition, the five-membered pyrazole analogue (**9h**) did not provide any better potency. The terminal phenyl group proved to be very critical for potency, though no specific interaction(s) (e.g., π–π interaction or hydrophobic interactions) could be identified from the complex structure. On the other hand, the internal phenyl ring was observed to form a π–cation interaction with Arg322 and a π–π stacking with Trp389. Thus, introduction of a nitrogen atom into this phenyl ring (**9i** and **9j**) resulted in significant decreases in potencies, likely due to reduced ability to form π–cation and π–π stacking interactions. The SAR studies were then focused on substitutions on the bi-phenyl rings. Introduction of a methoxy group to the *ortho*-position of the internal phenyl ring (**9k**) resulted in an improved potency (pIC_{50}: 8.1) comparing to the **4a** (pIC_{50}: 7.6). Moving the methoxyl substitution to the *meta*-position (**9l**) caused a slight decrease in potency (pIC_{50}: 7.5). Different *ortho*-susbstitutions with a variety of electronic properties including methyl (**9m**), fluoro (**9n**), and cyano (**9o**) groups were then explored and the methyl analogue (**9m**) proved to be the most potent (pIC_{50}: 8.4). Substitutions on the terminal phenyl ring were also evaluated. Introduction of a carboxylic acid group at *para*-position (**9p**) provided ~5-fold increase of potency (pIC_{50}: 8.3) compared with the parent compound (**4a**), probably due to ion pair interactions of the carboxylic acid group of **9p** with Arg396 of PHD2 enzyme. The acetic acid analogue (**9q**) demonstrated slightly decreased potency (pIC_{50}: 7.8). Moving the carboxylic acid to the *meta*-position (**9r**) resulted in 10-fold decrease in potency (pIC_{50}: 7.3) compared with **9p**, suggesting a less optimal polar interaction. The *ortho*-substituted analogue (**9s**) was totally inactive.

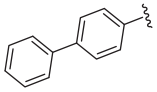
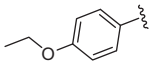
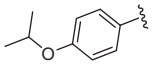
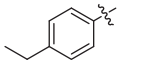
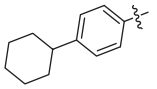
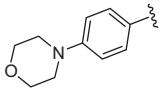
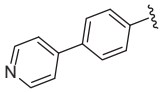
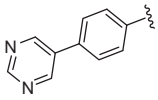
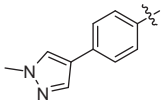
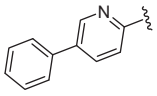
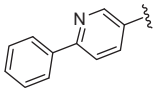
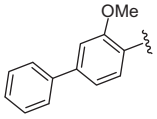
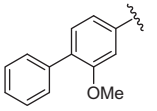
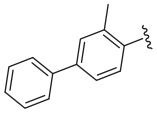
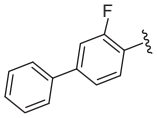
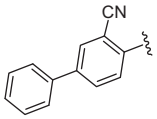
2.4. Pharmacokinetics

Between the most potent compounds discovered (**9m** and **9p**), compound **9p** was considered more developable due to its better physicochemical properties such as lower lipophilicity (*c*Log*P*: 2.2) compared with that of **9m** (*c*Log*P*: 4.8). Compound **9p** was thus evaluated in the mouse short oral absorption (SOA) study. To our delight, **9p** demonstrated good oral bioavailability with high peripheral exposure (DNAUC: 634 (ng h/mL)/(mg/kg)). In addition, low hepatic extraction (E_H = 0.10) for **9p** was observed, indicating low liver clearance of the compound.

Table 2
SAR on the left-hand-side biphenyl moiety (**9**)

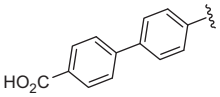
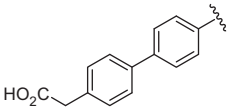
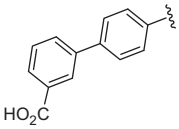
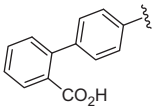


9

Compound	R	PHD2 pIC_{50}[a]	*c*Log*P*[b]
4a		7.6	4.75
9a		5.7	3.31
9b		5.9	3.62
9c		5.4	3.89
9d		6.0	5.48
9e		5.5	2.32
9f		6.4	3.25
9g		5.5	2.29
9h		6.3	2.86
9i		5.7	3.25
9j		6.2	3.46
9k		8.1	4.67
9l		7.5	4.11
9m		8.4	4.85
9n		7.6	4.72
9o		7.4	4.18

(*continued on next page*)

Table 2 (*continued*)

Compound	R	PHD2 pIC_{50}[a]	*c*Log*P*[b]
9p		8.3	2.22
9q		7.8	1.75
9r		7.3	2.22
9s		<5	1.42

[a] PHD2 assay data is the average of at least two determinations.
[b] *c*Log*P* was calculated using the Daylight software.

3. Conclusion

In conclusion, we have discovered 2,8-diazaspiro[4.5]decan-1-one series as potent, orally bioavailable PHD2 inhibitors. To understand the binding mode of the series, the complex crystal structure of an analogue **4b** with the PHD2 enzyme was resolved. In our structure, the metal chelation interaction was maintained whereas the salt bridge with Arg383 of the enzyme was not observed. This is in contrast to all previous literature complex structure reports. Instead, compound **4b** was found to form several novel interactions with the enzyme. These interactions include a hydrogen bond with Arg322, a π–cation interaction with Arg322, a π–π stacking with Trp389, and a π–π stacking with His313. Guided by the structural information, extensive SAR studies were performed and compound **9p** was discovered with high enzymatic potency and a good oral in vivo PK profile.

4. Experimental

4.1. Chemistry Methods

Compounds not described below were purchased from commercial vendors or reported. Compounds' purity was determined using LC/MS analysis. Purification of compounds was carried out by conventional methods such as chromatography and/or recrystallization using suitable solvents. Chromatographic methods include column chromatography and MDAP (mass-directed auto-purification system).

^{1}H NMR spectral data were recorded on a Bruker 400 NMR spectrometer operating at 400 MHz. ^{13}C NMR spectra were recorded at 100 MHz. $CDCl_3$ is deuteriochloroform, DMSO-d_6 is hexadeuteriodimethylsulfoxide. Chemical shifts are given in parts per million (δ) downfield from the internal standard tetramethylsilane (TMS) or the NMR solvent. Abbreviations for NMR data are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, app = apparent, br = broad. *J* indicates the NMR coupling constant measured in Hertz. High resolution mass (HRMS) was operated in positive mode of electrospray ionization (ESI) at an orthogonal acceleration time-of-flight (oa-TOF), SYNAPT G2 HDMSTM (Waters, Manchester, UK). Solutions (500 ng/mL in acetonitrile: H_2O (1:1, v/v)) were introduced via infusion at a flow rate of 5 μL/min to acquire accurate mass. LCMS (Agilent 1200SL-6110) analysis was conducted for all assayed compounds on either acidic or basic conditions: (1) acidic condition refers to water containing 0.05% TFA/acetonitrile as mobile phase on Agilent SB-C18 column (1.8 μm, 4.6 × 30 mm) with MS and photodiode array detector (PDA). The following conditions were used: a gradient from 5% to 95% in 4 min (or 6 min) and held at 95% for 1 min; UV detection at 214 and 254 nm; a flow rate of 1.5 mL/min; full scan; mass range from 100 to 1000 amu; (2) basic condition refers to water containing 10 mM NH_4HCO_3 aqueous/acetonitrile as mobile phase on Waters XBridge C18 column (3.5 μm, 4.6 × 50 mm) with MS and photodiode array detector (PDA). The following conditions were used: a gradient from 5% to 95% in 5 min and held at 95% for 1 min; UV detection at 214 and 254 nm; a flow rate of 2 mL/min; full scan; mass range from 100 to 1000 amu. All the assayed compounds possess ⩾95% purity determined using LC/MS analysis. Column chromatography was performed on Isco or Biotage using a pre-packed silica gel column, a detector with UV wavelength at 254 nm and mixed solvents. MDAP equipped with 2489 UV detector, 2767 sample manager, 2545 pump and 3100 single quadrupole mass spectrometer was performed on Sunfire Prep C18 column (5 μm, 19 × 50 mm) using water containing 0.05% TFA/acetonitrile as mobile phase. The following condition was used: a gradient from 5% to 95% in 8 min and held in 95% for 2 min; a flow rate 30 mL/min.

4.2. General procedure for the preparation of compounds 4a–l

4.2.1. *tert*-Butyl 2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decane-8-carboxylate (7)

A mixture of *tert*-butyl 1-oxo-2,8-diazaspiro[4.5]decane-8-carboxylate **5** (200 mg, 0.79 mmol) and 4-iodo-1,1′-biphenyl **6** (337 mg, 1.2 mmol) in acetonitrile (10 mL) at 40 °C was degassed with stream of nitrogen for 20 min. Then K_2CO_3 (326 mg, 2.36 mmol), copper(I) iodide (37.4 mg, 0.20 mmol) and *N1*,*N2*-dimethylethane-1,2-diamine (20 mg, 0.23 mmol) were added sequentially. The reaction mixture was heated at 80 °C for 15 h. After cooling, the solution was diluted with ethyl acetate and washed with 0.1 M HCl aqueous solution. The organic layer was concentrated and purified by column chromatography to afford compound **7** (115 mg, 36%) as a white solid. MS (ES): $C_{21}H_{23}N_2O_3$ $(M-t\text{Bu}+H)^+$ calcd 351.2; found 351.1. LC/MS: t_R = 3.95 min, >95%, *m*/*z*: 351.1 $(M-t\text{Bu}+H)^+$.

4.2.2. 2-([1,1′-Biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one (8)

To *tert*-butyl 2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decane-8-carboxylate **7** (115 mg, 0.28 mmol) was added HCl (4.0 M in MeOH) (10 mL, 27.4 mmol). The reaction mixture was stirred at room temperature for overnight then was concentrated under reduced pressure to afford compound **8** (157 mg) as a HCl salt. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.83–1.95 (m, 2H), 2.15 (ddd, *J* = 4.0, 10.0, 14.4 Hz, 2H), 2.24 (t, *J* = 7.0 Hz, 2H), 3.21 (ddd, *J* = 3.5, 9.7, 12.9 Hz, 2H), 3.55 (ddd, *J* = 4.3, 6.1, 12.9 Hz, 2H), 3.96 (t, *J* = 7.0 Hz, 2H), 7.30–7.37 (m, 1H), 7.43 (t, *J* = 7.7 Hz, 2H), 7.62 (d, *J* = 7.3 Hz, 2H), 7.64–7.70 (m, *J* = 8.8 Hz, 2H), 7.70–7.76 (m, *J* = 8.8 Hz, 2H). MS (ES): $C_{20}H_{23}N_2O$ $(M+H)^+$ calcd 307.2; found 307.2. LC/MS: t_R = 2.44 min, >95%, *m*/*z*: 307.2 $(M+H)^+$.

4.2.3. 2-([1,1′-Biphenyl]-4-yl)-8-((3-methylpyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4a)

To a solution of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (HCl salt, 100 mg, 0.29 mmol) and 3-methylpicolinaldehyde (70.7 mg, 0.58 mmol) in DCM (10 mL) was added sodium triacetoxyborohydride (371 mg, 1.75 mmol). The reaction mixture was stirred at room temperature for overnight. The reaction was

quenched with water (0.2 mL) and the reaction mixture was concentrated. The residue was purified via MDAP to afford compound **4a** (41 mg, 27%) as a TFA salt. ^{1}H NMR (400 MHz, MeOH-d_4) δ ppm 1.87 (d, J = 14.56 Hz, 2H), 2.13–2.25 (m, 4H), 2.33 (s, 3H), 3.32 (t, J = 6.78 Hz, 2H), 3.58 (d, J = 12.30 Hz, 2H), 3.90 (t, J = 6.78 Hz, 2H), 4.58 (s, 2H), 7.31–7.42 (m, 2H), 7.46 (t, J = 7.65 Hz, 2H), 7.64–7.78 (m, 5 H), 7.78–7.83 (m, 2H), 8.53 (d, J = 3.76 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 176.3, 149.4, 146.8, 139.9, 139.3, 136.4, 132.6, 129.4, 127.8, 127.3, 126.8, 124.1, 120.4, 57.2, 49.8, 45.0, 29.5, 17.7. HRMS $C_{27}H_{30}N_3O$ (M+H)$^+$ calcd 412.2389, found: 412.2395. LC/MS: t_R = 2.96 min, 96.6%, m/z: 412.1 (M+H)$^+$.

4.2.4. 2-([1,1′-Biphenyl]-4-yl)-8-((1-methyl-1*H*-imidazol-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4b)

To a solution of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (HCl salt, 57 mg, 0.17 mmol) and 1-methyl-1*H*-imidazole-2-carbaldehyde (36.7 mg, 0.33 mmol) in DCM (10 mL) was added sodium triacetoxyborohydride (211 mg, 1.0 mmol). The solution was stirred at room temperature for overnight then the reaction was quenched with MeOH (0.2 mL). The reaction mixture was concentrated. The residue was purified via MDAP to afford compound **4b** (36 mg, 41%) as a TFA salt. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.65–1.78 (m, 2H), 1.89–2.03 (m, 2H), 2.12 (t, J = 6.40 Hz, 2H), 2.87–2.90 (m, 1H), 3.30–3.33 (m, 2H), 3.81 (s, 3H), 3.87 (t, J = 6.90 Hz, 2H), 4.22–4.27 (m, 3H), 7.32–7.38 (m, 1H), 7.40–7.42 (m, 1H), 7.46 (t, J = 7.65 Hz, 2H), 7.53–7.57 (m, 1H), 7.64–7.74 (m, 4H), 7.76–7.81 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 176.9, 139.9, 139.3, 136.2, 129.4, 127.7, 127.3, 126.8, 124.4, 120.3, 50.5, 49.5, 45.0, 43.5, 34.3, 30.9. HRMS $C_{25}H_{29}N_4O$ (M+H)$^+$ calcd 401.2341, found: 401.2335. LC/MS: t_R = 2.53 min, 100%, m/z: 401.2 (M+H)$^+$.

4.2.5. 2-([1,1′-Biphenyl]-4-yl)-8-benzyl-2,8-diazaspiro[4.5]decan-1-one (4c)

To a solution of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (TFA salt, 27 mg, 0.064 mmol) and benzaldehyde (6.82 mg, 0.064 mmol) in dichloromethane (20 mL) was added sodium triacetoxyborohydride (40.8 mg, 0.193 mmol) at room temperature. The reaction mixture was stirred at room temperature for overnight. The reaction was quenched with MeOH (200 μL). The reaction mixture was concentrated and the residue was by MDAP to afford **4c** (16 mg, 46.4%) as a TFA salt as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.73–1.91 (m, 2H), 1.91–2.10 (m, 2H), 2.18 (t, J = 6.8 Hz, 2H), 3.05–3.21 (m, 2H), 3.45–3.49 (m, 2H), 3.82–3.95 (m, 2H), 4.29–4.46 (m, 2H), 7.30–7.40 (m, 1H), 7.40–7.53 (m, 7H), 7.62–7.85 (m, 6H), 9.50 (s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 176.2, 139.9, 139.2, 136.3, 131.9, 131.7, 130.2, 130.1, 129.4, 127.8, 127.3, 126.8, 120.5, 120.3, 59.6, 48.6, 45.0, 43.7, 29.4, 27.4. HRMS $C_{27}H_{29}N_2O$ (M+H)$^+$ calcd 397.2280, found: 397.2292. LC/MS: t_R = 2.95 min, 100%, m/z: 397.2 (M+H)$^+$.

4.2.6. 2-([1,1′-Biphenyl]-4-yl)-8-((4-methylpyridin-3-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4d)

To a solution of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (TFA salt, 25 mg, 0.059 mmol) and 4-methylnicotinaldehyde (8.64 mg, 0.071 mmol) in dichloromethane (3 mL) and *N*,*N*-dimethylformamide (1 mL) was added sodium triacetoxyborohydride (12.6 mg, 0.059 mmol) at room temperature. The reaction mixture was stirred at room temperature for overnight. Water (10 mL) was added to the reaction mixture and then the mixture was extracted with DCM (2 × 10 mL). The combined fractions were dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by MDAP to give **4d** (4.9 mg, 15.8% yield) as a TFA salt. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.83 (d, 2H), 1.96–2.10 (m, 2H), 2.22 (t, J = 6.65 Hz, 2H), 3.27 (br s, 2H), 3.43 (br s, 2H), 3.90 (t, J = 6.78 Hz, 2H), 4.45 (br s, 2H), 7.32–7.38 (m, 1H), 7.41–7.49 (m, 3H), 7.64–7.74 (m, 4H), 7.76–7.82 (m, 2H), 8.55 (d, J = 5.02 Hz, 1H), 8.68 (s, 1H). HRMS $C_{27}H_{30}N_3O$ (M+H)$^+$ calcd 412.2389, found: 412.2396. LC/MS: t_R = 2.35 min, 100%, m/z: 412.3 (M+H)$^+$.

4.2.7. 2-([1,1′-Biphenyl]-4-yl)-8-((2-methylpyridin-3-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4e)

To a solution of 2-methylnicotinaldehyde (6.92 mg, 0.057 mmol) and 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (TFA salt, 20 mg, 0.048 mmol) in dichloromethane (DCM) (3 mL) and *N*,*N*-dimethylformamide (1 mL) was added sodium triacetoxyborohydride (10.08 mg, 0.048 mmol) at room temperature. The reaction mixture was stirred at room temperature for overnight. Water (10 mL) was added to the reaction mixture and then the mixture was extracted with DCM (2 × 10 mL). The combined fractions were dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by MDAP to give **4e** (2.8 mg, 11.1% yield) as a TFA salt. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.82 (d, 2H), 2.04 (t, J = 12.30 Hz, 2H), 2.22 (br s, 2H), 2.68 (s, 3H), 3.19–3.31 (m, 2H), 3.43 (br s, 2H), 3.90 (t, J = 6.53 Hz, 2H), 4.45 (br s, 2H), 7.31–7.38 (m, 1H), 7.46 (t, J = 7.65 Hz, 2H), 7.48–7.55 (m, 1H), 7.64–7.73 (m, 4H), 7.76–7.82 (m, 2H), 8.07 (d, J = 7.03 Hz, 1H), 8.59–8.65 (m, 1H). HRMS $C_{27}H_{30}N_3O$ (M+H)$^+$ calcd 412.2389, found: 412.2391. LC/MS: t_R = 2.27 min, 99.4%, m/z: 412.2 (M+H)$^+$.

4.2.8. 2-([1,1′-Biphenyl]-4-yl)-8-((3-methylpyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4f)

To a solution of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (TFA salt, 27 mg, 0.064 mmol) and picolinaldehyde (6.88 mg, 0.064 mmol) in DCM (20 mL) was added sodium triacetoxyborohydride (40.8 mg, 0.19 mmol). The reaction mixture was stirred at room temperature for overnight. The reaction was quenched with MeOH (0.2 mL) and the mixture was concentrated. The residue was purified by MDAP to afford compound **4f** (12 mg, 37%) as a TFA salt as an off-white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ ppm 1.84 (d, J = 13.55 Hz, 2H), 2.06–2.22 (m, 4H), 3.22–3.26 (m, 2H), 3.49 (d, J = 12.05 Hz, 2H), 3.88 (t, J = 6.59 Hz, 2H), 4.53 (s, 2H), 7.30–7.39 (m, 1H), 7.46 (t, J = 7.34 Hz, 2H), 7.50 (t, J = 6.02 Hz, 1H), 7.56 (d, J = 7.53 Hz, 1H), 7.67 (d, J = 7.53 Hz, 2H), 7.71 (m, J = 7.91 Hz, 2H), 7.79 (m, J = 7.91 Hz, 2H), 7.95 (t, J = 7.72 Hz, 1H), 8.67–8.74 (m, 1H), 10.00 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 176.2, 150.9, 150.1, 139.9, 139.2, 138.2, 136.3, 129.4, 127.8, 127.3, 126.9, 125.4, 124.7, 120.4, 59.9, 49.3, 45.0, 40.6, 29.4. HRMS $C_{26}H_{28}N_3O$ (M+H)$^+$ calcd 398.2232, found: 398.2246. LC/MS: t_R = 2.66 min, 100%, m/z: 398.1 (M+H)$^+$.

4.2.9. 2-([1,1′-Biphenyl]-4-yl)-8-((3-chloropyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4g)

A solution of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (30 mg, 0.098 mmol) and 3-chloropicolinaldehyde (20.8 mg, 0.15 mmol) in DCM (3 mL) and DMF (1 mL) was stirred at room temperature for 30 min. Sodium triacetoxyborohydride (31 mg, 0.15 mmol) was then added. The reaction mixture was stirred at room temperature for overnight. The reaction was quenched with water (0.2 mL) and the reaction mixture was extracted with DCM (20 mL). The organic fraction was separated and dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by MDAP to afford compound **4g** (7.5 mg, 14%) as a TFA salt. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.84 (d, J = 13.55 Hz, 2H), 2.14–2.27 (m, 4H), 3.30–3.38 (m, 2H), 3.61 (d, J = 11.29 Hz, 2H), 3.90 (t, J = 6.65 Hz, 2H), 4.73 (s, 2H), 7.32–7.39 (m, 1H), 7.46 (t, J = 7.65 Hz, 2H), 7.57 (dd, J = 8.28, 4.77 Hz, 1H), 7.64–7.75 (m, 4H), 7.77–7.84 (m, 2H), 8.12 (dd, J = 8.03, 1.25 Hz, 1H), 8.69 (d, J = 3.76 Hz, 1H),

10.00 (br s, 1H). HRMS $C_{26}H_{27}ClN_3O$ $(M+H)^+$ calcd 432.1843, found: 432.1845. LC/MS: t_R = 2.71 min, 99.9%, *m*/*z*: 432.0 $(M+H)^+$.

4.2.10. 2-([1,1′-Biphenyl]-4-yl)-8-((3-(trifluoromethyl)pyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4h)

A solution of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (TFA salt, 30 mg, 0.071 mmol) and 3-(trifluoromethyl)picolinaldehyde (18.74 mg, 0.107 mmol) in DCM (3 mL) and DMF (1 mL) was stirred at room temperature for 30 min. Sodium triacetoxyborohydride (22.7 mg, 0.11 mmol) was then added. The reaction mixture was stirred at room temperature for overnight. The reaction was quenched with water (0.2 mL) and the reaction mixture was extracted with DCM (20 mL). The organic fraction was separated and dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by MDAP to afford compound **4h** (13.5 mg, 33%) as a TFA salt. ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.82 (d, *J* = 13.80 Hz, 2H), 2.17–2.29 (m, 4H), 3.27–3.45 (m, 2H), 3.51–3.59 (m, 2H), 3.90 (t, *J* = 6.53 Hz, 2H), 4.80 (s, 2H), 7.31–7.39 (m, 1H), 7.46 (t, *J* = 7.53 Hz, 2H), 7.63–7.77 (m, 5 H), 7.77–7.85 (m, 2H), 8.36 (d, *J* = 8.28 Hz, 1H), 9.00 (d, *J* = 4.27 Hz, 1H), 10.07 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 153.0, 139.8, 139.2, 136.3, 129.5, 127.8, 127.3, 126.8, 124.4, 120.3, 49.8, 45.0, 43.6, 29.4, 27.6. HRMS $C_{27}H_{27}F_3N_3O$ $(M+H)^+$ calcd 466.2106, found: 466.2112. LC/MS: t_R = 2.94 min, 99.9%, *m*/*z*: 466.0 $(M+H)^+$.

4.2.11. 2-([1,1′-Biphenyl]-4-yl)-8-((3-methoxypyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (4i)

To a mixture of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (TFA salt, 100 mg, 0.24 mmol) and 3-methoxypicolinaldehyde (100 mg, 0.73 mmol) in dichloromethane (5 mL) and *N*,*N*-dimethylformamide (5 mL) was added three drops of acetic acid. The reaction mixture was stirred at room temperature for overnight. Sodium triacetoxyborohydride (151 mg, 0.71 mmol) was added to the reaction mixture. The reaction mixture was stirred at room temperature for overnight. The reaction mixture was extracted with DCM (2 × 20 mL). The organic fractions were combined and the combined solution was dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by MADP to give compound **4i** (15.5 mg, 15%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.46 (d, *J* = 12.55 Hz, 2H), 1.73 (td, *J* = 12.61, 3.64 Hz, 2H), 2.01 (t, *J* = 6.90 Hz, 2H), 2.13–2.23 (m, 2H), 2.83 (d, *J* = 11.80 Hz, 2H), 3.60 (s, 2H), 3.78–3.84 (m, 5 H), 7.26–7.37 (m, 2H), 7.39–7.48 (m, 3H), 7.67 (t, *J* = 8.16 Hz, 4H), 7.74–7.81 (m, 2H), 8.10 (d, *J* = 4.52 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 178.0, 154.7, 147.4, 140.5, 140.0, 139.6, 135.9, 129.4, 127.7, 127.2, 126.8, 123.8, 120.1, 118.6, 58.6, 56.1, 49.9, 44.9, 44.6, 32.4, 28.4. HRMS $C_{27}H_{30}N_3O_2$ $(M+H)^+$ calcd 428.2338, found: 428.2347. LC/MS: t_R = 2.78 min, 100%, *m*/*z*: 428.2 $(M+H)^+$.

4.2.12. 2-(6-((2-([1,1′-Biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)acetic acid (4j)

A mixture of 2-([1,1′-biphenyl]-4-yl)-2,8-diazaspiro[4.5]decan-1-one **8** (TFA salt, 500 mg, 1.19 mmol), 5-bromo-3-methylpicolinaldehyde (285 mg, 1.43 mmol), and sodium acetate (98 mg, 1.19 mmol) in DCM (20 mL) and DMF (4 mL) was added five drops of HOAc. The reaction mixture was stirred at room temperature for 16 h. To the mixture was then added sodium triacetoxyhydroborate (250 mg, 1.19 mmol). After stirring for 1 h, more sodium triacetoxyhydroborate (250 mg, 1.19 mmol) was added, and the resultant mixture was stirred for another 2 h. Saturated aqueous $NaHCO_3$ solution (20 mL) was added. The organic fraction was separated and dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by column chromatography to afford 2-([1,1′-biphenyl]-4-yl)-8-((5-bromo-3-methylpyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (437 mg, 74.9%). MS (ES): $C_{27}H_{29}BrN_3O$ $(M+H)^+$ calcd 490.1, found: 490.2.

To a dried three-necked flask were added 2-([1,1′-biphenyl]-4-yl)-8-((5-bromo-3-methylpyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (100 mg, 0.20 mmol) and THF (5 mL) under nitrogen. The mixture was heated to reflux and it became a clear solution. To the mixture were added tri-*tert*-butylphosphine (TFA salt, 23.7 mg, 0.082 mmol), cesium carbonate (26.6 mg, 0.082 mmol), $Pd_2(dba)_3$ (28.0 mg, 0.031 mmol) and (2-ethoxy-2-oxoethyl)zinc(II) bromide (474 mg, 2.04 mmol). Then the reaction mixture was stirred at reflux for 30 min. After cooling, saturated aqueous NH_4Cl solution (15 mL) was added. The aqueous fraction was separated and extracted with DCM (20 mL). The combined organic fractions were dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by column chromatography to afford ethyl 2-(6-((2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)acetate (101 mg, 100%). MS (ES): $C_{31}H_{36}N_3O_3$ $(M+H)^+$ calcd 498.3, found: 498.3.

To a solution of ethyl 2-(6-((2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)acetate (101 mg, 0.20 mmol) in THF (10 mL), ethanol (5 mL) and water (2 mL) was added KOH (11.4 mg, 0.20 mmol). The reaction mixture was stirred at room temperature for 3 h. Then the mixture was acidified to pH 6 using 2 N HCl aqueous solution. The organic solvents were evaporated under reduced pressure. The resultant aqueous solution was extracted with DCM (2 × 20 mL). The organic fractions were combined and dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by MDAP to afford **4j** (9 mg, 9.6%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.44–1.61 (m, 2H), 1.68–1.87 (m, 2H), 2.02–2.30 (m, 4H), 2.38 (s, 3H), 2.68–2.92 (m, 2H), 3.54–3.74 (m, 4H), 3.83 (t, *J* = 6.78 Hz, 2H), 7.31–7.37 (m, 1H), 7.42–7.51 (m, 3H), 7.63–7.72 (m, 4H), 7.75–7.81 (m, 2H), 8.20 (s, 1H), 12.49 (br s, 1H). ^{13}C NMR (100 MHz, DMSO-d_6) δ ppm 172.8, 156.0, 146.9, 139.9, 139.5, 132.8, 129.4, 127.7, 127.3, 126.8, 120.2, 48.0, 45.0, 37.7, 32.1, 28.4, 18.3. HRMS $C_{29}H_{32}N_3O_3$ $(M+H)^+$ calcd 470.2444, found: 470.2451. LC/MS: t_R = 2.69 min, 100%, *m*/*z*: 470.3 $(M+H)^+$.

4.2.13. 3-(6-((2-([1,1′-Biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)propanoic acid (4k)

To a dried three-neck flask was added 2-([1,1′-biphenyl]-4-yl)-8-((5-bromo-3-methylpyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (150 mg, 0.31 mmol) and THF (5 mL) under nitrogen. The mixture was heated to reflux and it became a clear solution. To the mixture were added tri-*tert*-butylphosphine (TFA salt, 35.5 mg, 0.122 mmol), cesium carbonate (40.0 mg, 0.12 mmol), $Pd_2(dba)_3$ (42.0 mg, 0.046 mmol), and (3-ethoxy-3-oxopropyl)zinc(II) bromide (6.12 mL, 3.1 mmol). The reaction mixture was stirred at reflux for 30 min. After cooling, saturated aqueous NH_4Cl solution (10 mL) was added. The mixture was extracted with DCM (2 × 20 mL). The organic fractions were combined and the combined solution was dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by column chromatography to afford ethyl 3-(6-((2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl-5-methylpyridin-3-yl)propanoate (63.7 mg, 40.7%). MS (ES): $C_{32}H_{38}N_3O_3$ $(M+H)^+$ calcd 512.3, found: 512.3.

To a solution of ethyl 3-(6-((2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)propanoate (63.7 mg, 0.124 mmol) in THF (10 mL), ethanol (10 mL) and water (2 mL) was added KOH (2 ml, 6.00 mmol, 3 M in water).

The reaction mixture was stirred at room temperature for 2 h. Then the mixture was acidified to pH 5 by addition of 2 N HCl aqueous solution. The mixture was extracted with DCM (2 × 20 mL). The organic fractions were combined and dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by MDAP to afford **4k** (11.3 mg, 18.9%). ^{1}H NMR (400 MHz, DMSO-d_6) δ ppm 1.47 (d, J = 12.55 Hz, 2H), 1.72 (t, J = 10.92 Hz, 2H), 2.03–2.09 (m, 2H), 2.15 (t, J = 11.67 Hz, 2H), 2.37 (s, 3H), 2.55 (t, J = 7.53 Hz, 2H), 2.65–2.75 (m, 2H), 2.75–2.81 (m, 2H), 3.56 (s, 2H), 3.82 (t, J = 6.90 Hz, 2H), 7.31–7.37 (m, 1H), 7.41–7.48 (m, 3H), 7.63–7.71 (m, 4H), 7.78 (d, J = 8.78 Hz, 2H), 8.16 (s, 1H), 12.18 (br. s, 1H). HRMS $C_{30}H_{34}N_3O_3$ $(M+H)^+$ calcd 484.2600, found: 484.2602. LC/MS: t_R = 2.77 min, 100%, m/z: 484.2 $(M+H)^+$.

4.2.14. 4-(6-((2-([1,1′-Biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)butanoic acid (4l)

To a dried three-necked flask were added 2-([1,1′-biphenyl]-4-yl)-8-((5-bromo-3-methylpyridin-2-yl)methyl)-2,8-diazaspiro[4.5]decan-1-one (100 mg, 0.20 mmol) and THF (5 mL) under nitrogen. The mixture was heated to reflux and it became a clear solution. To the mixture were added tri-*tert*-butylphosphine (TFA salt, 23.7 mg, 0.082 mmol), cesium carbonate (26.6 mg, 0.082 mmol), $Pd_2(dba)_3$ (28.0 mg, 0.031 mmol), and (4-ethoxy-4-oxobutyl)zinc(II) bromide (0.41 ml, 0.20 mmol). The reaction mixture was stirred at reflux for 30 min. After cooling, saturated aqueous NH_4Cl solution (10 mL) was added. The mixture was extracted with DCM (2 × 20 mL). The organic fractions were combined and the combined solution was dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue was purified by column chromatography to afford ethyl 4-(6-((2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)butanoate (71 mg, 67.6%). MS (ES): $C_{33}H_{40}N_3O_3$ $(M+H)^+$ calcd 526.3, found: 526.3.

To a solution of ethyl 4-(6-((2-([1,1′-biphenyl]-4-yl)-1-oxo-2,8-diazaspiro[4.5]decan-8-yl)methyl)-5-methylpyridin-3-yl)butanoate (71 mg, 0.135 mmol) in tetrahydrofuran (THF) (10 mL), ethanol (5 mL) and water (2 mL) was added 3 M potassium hydroxide aqueous solution (2 mL, 6.00 mmol). The reaction mixture was stirred at room temperature for 3 h. Then the mixture was acidified by addition of 2 N HCl aqueous solution to pH 6.0. The mixture was extracted with DCM (2 × 20 mL). The organic fractions were combined and combined solution was dried over anhydrous sodium sulfate. The dried solution was filtered and the filtrate was concentrated. The residue purified by MDAP to give **4l** (1.2 mg, 1.5% yield) as a TFA salt MS $C_{31}H_{36}N_3O_3$ $(M+H)^+$ calcd 498.28, found: 498.3. LC/MS: t_R = 2.77 min, 100%, m/z: 498.3 $(M+H)^+$.

4.3. Crystallography

Purification of human PHD2 is as described previously.[15] PHD2 inhibitor complex was made by mixing purified PHD2 and inhibitor compound at 1:2 molar ratio. Co-crystals of PHD2 complexed with a 100-fold weaker PHD inhibitor compound (unpublished GSK proprietary data) grew spontaneously by mixing 2 μL of PHD2 complex (20 mg/mL) and 2 μL of a reservoir solution (30% PEG 3350, 200 mM ammonium sulfate, 100 mM sodium acetate, pH 4.8) in sitting drop vapor diffusion method at 20 °C. Structure of this complex was solved and revealed a clean empty pocket (unpublished GSK proprietary data). Co-crystals of PHD2 and this proprietary compound were transferred to a fresh drop of reservoir solution without inhibitor compound before 1 mM of compound **4b** was introduced to the transfer drop and soaking was allowed for 2 days at 20 °C. Soaked crystals were quickly transferred to a drop of reservoir solution supplemented with 1 mM inhibitor compound and 10% glycerol before freezing in liquid nitrogen.

X-ray diffraction data were collected at beamline 17U at the Shanghai Synchrotron Radiation Facility and were processed using HKL2000.[20] The complex crystallized in the space group with unit cell dimensions of a = 111.455, b = 111.455, c = 39.974, and γ = 120, and one complex per asymmetric unit. The structure was solved by molecular replacement using the apo PHD2 structure (GSK proprietary) as a search model. Model refinement was done against full range of data using REFMAC5[21] and rebuilt using COOT.[22] The model was refined to a final R_{work} = 23.0 and R_{free} = 25.9 and contained 1 copy of PHD2, 1 copy of compound **4b**, and water molecules. The bond length and angle root-mean-square deviation from ideality are 0.008 Å and 1.388°, respectively.

The coordinates of the PHD2 complex with compound **4b** have been deposited in the Protein Data Bank with the access number 4JZR.

4.4. Docking

Ligand was prepared using LigPrep [Schrödinger 2012R1] to generate tautomers at pH 7 (using Epik) and steoreoisomers. Receptor was prepared using Protein Preparation Wizard [Schrödinger 2012R1] to assign bond orders, add hydrogen atoms, refine h-bond assignment, and perform energy minimization, etc. Then docking grid was generated around compound **4b** in the crystal structure using Glide [Schrödinger 2012R1]. Eventually ligand was docked using Glide with two constraints: h-bond with Arg383 and h-bond with Arg322. All other settings took the default values.

As compound **4k** is large with the biphenyl which may hinder conformational sampling during docking, we replaced the biphenyl with a methyl before docking and added it back to the top docking pose afterwards.

4.5. Homogeneous time resolved fluorescence (HTRF) assay for measurement of prolyl hydroxylase domain 2 (PHD2) activity

10 nL of compound was shot by Echo (Labcyte Inc.) into NUNC 384-well Black PP Plate. 5 μL of enzyme working solution (assay buffer (50 mM HEPES, 50 mM KCl, pH 7.5) with 1 mM TCEP HCl, 0.2 mg/mL BSA, 5 μg/mL $FeCl_2$, 0.4 mM ascorbic acid, 0.3 mM CHAPS and 16 nM His-streptag-tev-EGLN1) was added into compound wells and high control wells in compound-containing NUNC 384-well assay plate and 5 μL of control working solution (enzyme working solution without EGLN1 enzyme) was added into low control wells. The plate was then incubated at room temperature for 30 min. 5 μL of substrate working solution (assay buffer with 50 nM biotin-VBC, 1 μg/mL Europium/Streptavidin, 3.8 μM HIF1a-Cy5CODD, 1 μM 2-oxoglutaratic acid and 0.3 mM CHAPS) was added into all wells of assay plate. After another incubation at room temperature for 30 min, the plate was loaded into ViewLux imager (PerkinElmer) and read with LANCE protocol.

4.6. Short oral absorption (SOA) study in male mice

All animal studies were ethically reviewed and carried out in accordance with European Directive 2010/63/EU and the GSK Policy on the Care, Welfare and Treatment of Animals.

Six male C57BL/6 mice received oral gavage administration (p.o.) of the test compound at 10 mg/kg. The dose was prepared on the day of administration in suspension in DMSO:1% methylcellulose (v/v, 1:99), at a concentration of 1 mg/mL (dose volume = 10 mL/kg). At 15 min, 30 min, 1 h, 3 h, 5 h and 7 h post p.o. administration, fifty microliters of blood were collected via portal vein and cardiac puncture under anesthesia, respectively, and were diluted

with 150 μL of water. There was one animal for each time point. Blood sample was stored at −80 °C until LC/MS/MS bioanalysis. The measured concentrations were corrected for dilution factor, and then the peripheral exposure was determined based on the area under the curve (AUC) systemic blood concentrations. Hepatic extraction (E_H) was calculated based on the AUC of systemic and hepatic portal vein blood concentrations:

$$E_H = 1 - \frac{AUC_{SYS}}{AUC_{HPV}}$$

Acknowledgment

We thank Drs. Kelly Dong and Ruina Gao for measuring HRMS data.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.08.046.

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