Electronic Supplementary Information

Azole Derivatives as Novel Non-iron-chelating inhibitors of Prolyl Hydroxylase 3 for HIF-1 activation

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1 Experimental Section

1.1 General

Expression host, *E. coli* BL21 (DE3) pLysS and pET32α(+)Vector were obtained from Novagen (Germany). Isopropyl β-D-thiogalactopyranoside (IPTG), 2-oxoglutarate, ascorbate, bovine serum albumin (BSA), dithiothreitol (DTT) and catalase were from Sigma (USA). HIF-1α peptide corresponding to residues 556–574(DLDLEMLAPYIPMDDDFQL) was synthesized by Shanghai Apeptide Co., Ltd. Sequencing grade modified trypsin was purchased from Promega (USA). All other reagents were of analytical grade and all solutions were prepared using Milli-Q deionized water. Azole compounds BI and BT were purchased from J& K Chemical (China). IPA, TPA, BIPA, BTPA, TEB and BEBT were synthesized in our group as reported.1-5

1.2 Protein expression and purification

The recombinant human PHD3 enzyme was expressed in *E. coli* as described previously, and purified by Ni-NTA6 and size exclusion chromatography. Gel filtration was performed on a Superdex 200 column (GE Healthcare) equilibrated with GF buffer (20 mM Na₂HPO₄, 0.5 M NaCl, 1 mM DTT, pH 6.5). A portion of 2 ml of the concentrated Ni-NTA fraction was applied to the column and eluted with GF buffer. The optimal flow rate for separation was 0.8 ml/min. The fractions containing PHD3 were collected and concentrated. The purity of the protein was identified by SDS-PAGE and MALDI-TOF-MS. The protein concentration was measured by BCA Protein Assay Kit. Approximately 90% of highly pure PHD3 was eluted as monomer form corresponding to 45 kDa. The reducing agents stabilized PHD3 throughout gel filtration.

1.3 Docking simulations

Docking studies were carried out using AutoDock Vina and VSDK Program.7, 8 Autodock Tools (ADT) was used for correcting the ligands and protein parameters.9 The 3D structures of the ligands were generated using ChemDraw Ultra 9.0 and Chem3D Ultra 9.0 (Cambridge Soft) and energetically minimized using the MOPAC feature within Chem3D Ultra 8.0.10 The simulated crystal structure of human PHD3 was estimated from the website.11-13 The computation automatically simulated PHD3 structure models based on the crystal structures of the catalytic domain of PHD2 (PDB ID: 3OUJ).11-14 The enzyme and ligand input structures were prepared under a GRID-based protocol with a rectangular grid box constructed over the PHD3 active site (54×48×64 Å³, 0.375 Å). Tyr132, His135, Asp137, Tyr151, His196 Arg205 and Trp211 were set as the flexible residues compared with the sequence of PHD2 according to the literature.15 Kollman united atom charges and desolvation parameters were assigned, nonpolar hydrogens were removed, and partial atomic charges were added using ADT. The charge of Fe (II) was manually set to +2.00 in the receptor PDBQT file. LigPlot⁺ was used to analyze the hydrogen bonds and hydrophobic interactions of the simulation results.16, 17

1.4 The fluorescence of PHD3 with titration of azole compounds

The protein fluorescence emission spectra were recorded by Thermo Scientific Varioskan Flash (Thermo Scientific, USA) at the excitation wavelength of 280 nm with a 96-well black plate at step size of 1 nm. The initial volume of each test protein solution was 100 μL. The slit widths on the excitation monochromators was 12 nm. All measurements were performed at 37 °C. The fluorescence was measured by titrating PHD3 (5 μM) and Fe²⁺ (50 μM) mixture or/and NOG
(50μM) in 50 mM PBS (pH 7.0) with increasing amounts of sixazole compounds (IPA, TPA, BIPA, BTPA, TEB and BEBT) in a final concentration range of 10⁻⁶ - 10⁻⁴ M. A blank spectrum of buffer was collected simultaneously for subtraction.

1.5 Enzyme inhibition assay

As presented before⁶, the PHD3 activity was assayed by mixing 1 mM DTT, 0.6 mg/mL catalase, 2 mM ascorbate, 2 mg/mL BSA, 50 μM FeCl₂ (prepared as 500 mM stock in 20 mM HCl and diluted with water), HIF 19 peptide, enzyme and 20 mM PBS, pH 7.0 to a final volume of 96 μL, and initiating the reaction by adding 4 μL of 2OG (160 μM). After activity and derivatization assays, the concentration of remained 2OG was quantitatively analyzed from the maximum response of the fluorescence intensity of the derivatization product between 2OG and OPD.¹⁸ Scanning emission and excitation spectra were recorded on a Thermo Scientific Varioskan Flash with a 96-well black plate at the step size of 1 nm (λₐₓ 340 nm, λₑₜₐ₉ 420 nm).

1.6 Circular dichroism measurements and secondary structure analysis

CD (200-250 nm) spectra were recorded on a JASCO-J810 spectropolarimeter (Jasco Co., Japan) in a cell of 1 mm slit width and 10 mm light length. The scanning rate was set at 50 nm•min⁻¹. The spectra were the average of two readings. Standard measurements were carried out at room temperature in diluted enzyme solutions (4 μM, pH 7.0). The protein spectrum was derived by automatically subtracting the blank spectrum of buffer collected previously. The secondary structure contents of PHD3 were estimated with the Jasco secondary structure manager software using the reference CD data-Yang. Jwr.¹⁹ The β-sheet contents were recalculated through an empirical equation describing the relationship between α-helix and β-sheet contents.²⁰

1.7 Limited Trypsinolysis

Sequencing grade modified trypsin was prepared in 15 mM NH₄OAc (pH 6.5) at 100 μg•mL⁻¹. PHD3 and 2OG were prepared in 15 mM NH₄OAc (pH 7.8). BIPA and BTPA were prepared in deionized water (Milli-Q), whereas TEB and BEBT were prepared to 100 mM in DMSO prior to dilution in deionized water. Fe²⁺ was prepared by dissolving diammonium iron(II) sulphate to 500 mM in 20 mM HCl prior to dilution with water. Trypsinolysis was carried out under the instructions previously reported.²¹ The final assay mixture composition typically contained 50 μM PHD3, 50 μM Fe²⁺, 500 μM 2OG or inhibitor and trypsin (trypsin/PHD3 1:50 w/w). Quenched digest samples were immediately stored at -80 °C until analysis.

1.8 MALDI-TOF-MS Analysis

Prior to analysis, quenched digest samples were thawed and kept on ice. A 10 mg•mL⁻¹ solution of recrystallized sinapinic acid (SA) was prepared in MeCN/H₂O/CF₃CO₂H (40:60:0.1) as supplied by the manufacturer. The digest sample (3 μL) and SA (3 μL) were mixed, 2 μL of which was then pipetted onto a MALDI plate spot and air-dried. Mass spectrometry analysis were conducted using an AB SCIEX MALDI TOF-TOF 4800 Analyzer (AB SCIEX, Shanghai, China) in linear middle mass positive-ion mode. Calibration was performed with Sequenenzyme peptide standard kit (AB SCIEX). The MS spectra were processed using TOF/TOF Series Explorer (AB SCIEX). The fixed laser energy was 4500 J for sample acquisition. The MS spectra were plotted based on the accumulation of at least 800 laser shots.

1.9 Cell culture
NCI-H446 cells (from the Cell Bank of the Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 (Hyclone) with 10% heat-inactivated fatal bovine serum (GIBCO), 100 units/mL penicillin, and 100 units/mL streptomycin (GIBCO) in a humidified 5% CO₂ environment at 37 °C. At 70%–80% confluence, the cells were harvested after being treated with 0.25% (w/v) trypsin-EDTA (Sigma-Aldrich), and then reseeded for expansion. 1×10⁶ H446 cells were seeded on 100 mm culture dishes in complete medium for 12 h, and then exposed to medium with 25 μM compound each for 12 h to extract the total cellular protein. H446 cells were plated onto 6-well culture plates (corning, USA) at a density of 4×10⁴ cells per well, incubated for 12 h in complete media, and then given flash medium with 25 μM compound each for 12 h. Culture supernatants were collected and stored at -80 °C for VEGF ELISA Assays, and the cells were harvested to extract RNA.

1.10 Cell proliferation Assay
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were applied to test cell viability. NCI-H446 cells (2.0 × 10⁵ cells) in a 96-well plate (corning, USA) were incubated for 12 h. Then the medium was renewed to that containing various concentrations of compound and incubated for 24 h. Subsequently, 20 μL of MTT (0.5 mg/mL final concentration) was added to each well. After incubation for 4 h, 150 μL of DMSO was added to the cultures to dissolve the crystals. The absorbance at 570 nm was recorded by an automatic enzyme-linked immunoassay plate reader (Thermo Scientific Varioskan Flash, USA).

1.11 Semi-quantitative Reverse Transcription-PCR Analysis
Total RNA was isolated from NCI-H446 cells using Trizol reagent by following the manufacturer’s instructions. The RNA concentration was quantified to two micrograms of each sample through Nanodrop ND-1000 (Thermo Scientific, USA). Each RNA sample was reversely transcribed into cDNA by PrimeScript reverse transcriptase using a PrimeScript RT-PCR kit. PCR proceeded using the cDNA as a template and TakaRa Taq™ kit by following the manufacturer’s instructions. The number of PCR cycles determined from the plot was 30 for HIF-1α, VEGF and GLUT-3 and 25 for β-actin. The amount of amplified product was detected by 0.1% agarose gel electrophoresis, scanned and analyzed using Quantity One (Bio-Rad, Hercules, CA, USA). The primers: HIF-1α-sense 5’-AGTGTACCCCTAACTAGCCG-3’, HIF-1α-antisense 5’-CACAAATCAGCCAAAGC-3’, VEGF-sense 5’-GAAGGAGGAGGCGAGAAT-3’, VEGF-antisense 5’-CACAGGATGGCTTGAAGAT-3’, GLUT-1-sense 5’-CATGCAGGTGTTCAAGAG-3’, GLUT-1-antisense 5’-GAGGCTACAAAGACCAA-3’, β-actin-sense 5’-GACCTGACTGACTACCT-3’, β-actin antisense 5’-TCTTCATTGTGCTGGGTGC-3’. All reagents were purchased from Takara.

1.12 Western blotting
Total cellular protein extracts were obtained at 4 °C in lysis buffer containing 20 mM Tris–HCl (pH8.0), 250 mM NaCl, 0.4 mM Na₂VO₃, 1% SDS and 1×Complete mini protease inhibitor cocktail tablets (Roche Diagnostics, Switzerland). Samples were separated by 12% SDS-PAGE and transferred to Immobilon-P transfer membrane (Millipore, USA). Membranes were blocked with 10% nonfat milk in TBS containing 0.1% Tween-20 at room temperature for 1 h, and then incubated with anti-β-Actin (1:2000, Cell Signalling), anti-HIF-1α (1:1000, BD Transduction Laboratories™) and anti-GLUT-1 (1:100, Santa Cruz Biotechnology), respectively. Antibodies
were diluted in TBS with 2% bovine serum albumin at 4 °C overnight. Blots were incubated with a HRP-conjugated anti-rabbit secondary (1:5000) antibody, an anti-mouse secondary (1:5000) antibody and an HRP-conjugated anti-goat secondary (1:5000) antibody for 1 h, respectively. Enhanced chemiluminescence (ECL, Cell Signalling) was performed afterwards. Blot images were scanned and analyzed using Quantity One (Bio-Rad, USA).

### 1.13 ELISA Assays

ELISA assays (human VEGF, Boster, China) were performed to determine the levels of VEGF protein from NCI-H446 cells using the cell culture supernatant. The detection was performed following the recommendations of the manufacturer.

### 1.14 Statistical Analysis

Data was expressed as mean ± standard derivation (SD) and analyzed with Excel 2010. Student’s two-tailed t-test was used to compare two independent groups. Statistical significance was defined as P<0.05.

### Supplementary Results

**Effects of the compounds on PHD3 fluorescence and calculation of binding affinity**

Compounds were titrated to PHD3-Fe$^{2+}$ complex and the fluorescence spectra were recorded. IPA, TPA, BIPA, BTPA, TEB and BEBT continually quenched the fluorescence of PHD3. The maximum fluorescence emission intense data was analyzed in terms of Hill equation (eqn(2)).

$$
\gamma = \frac{[L_f]^n}{(K_d^n + [L_f]^n)} \quad (1)
$$

where $\gamma$ is the ratio of the concentration of bound ligand to total available binding sites, $[L_f]$ is the free ligand concentration, $K_d$ is the dissociation constant for the PHD3-Fe complex and ligand, and $n$ is hill coefficient.$^{22,23}$ The fluorescence quenching data fitted well through non-linear regression of Hill equation (Fig. 4). The average apparent dissociation constant ($K_d$) values of PHD3-Fe complex binding with are $5.44 \times 10^{-5}$, $1.46 \times 10^{-3}$, $1.52 \times 10^{-5}$, $3.47 \times 10^{-6}$, $5.67 \times 10^{-6}$, and $7.34 \times 10^{-6}$ mol/L., respectively.

The effects of inhibitors on the fluorescence of PHD3-Fe$^{2+}$-NOG complex were then studied. The fluorescence emission intensity of the complex also been quenched by BIPA, BTPA, TEB and BEBT, while IPA and TPA failed to affected the fluorescence of the mixture regularly. Hill blot provides the average apparent dissociation constant ($K_d$) values of PHD3-Fe-NOG mixture with inhibitors binding as well. The $K_d$ values are estimated to be $1.74 \times 10^{-5}$, $1.42 \times 10^{-5}$, $2.02 \times 10^{-5}$ and $8.34 \times 10^{-5}$ μM, respectively.
Supplementary Figures

Fig. S1 Predicted structures of IPA (a), TPA (b), BIPA (c), TEB (d), BI (e) and BT (f) binding to catalytic center of PHD3. Docking simulation was carried out by AutoDock Vina. Docked conformation of ligand-protein interactions were shown by PyMOL. Iron is displayed in orange. Hydrogen bonds are shown in dashed lines (yellow). Residues that interacted with the ligand are shown as lines representation. The docked inhibitors are displayed as stick representation. The compound colors are shown by element (C, cyan; H, white; N, blue; O, red). Labels are shown in white.
Fig. S2 Analysis of hydrogen bonds and hydrophobic interactions of IPA (a), TPA (b), BIPA (c), TEB (d), BI (e) and BT (f) with residues in PHD3 active site by Ligplot+. The data were derived from molecular modeling results simulated by AutoDock Vina. Hydrogen bonds are shown as dashed lines (Olive green) and residues are labeled in green. Residues having hydrophobic interactions with the ligand are shown as lashes (Brick red) with their name labeling in dark, and hydrophobic “bonds” are shown as dotted lines (Brick red).
Fig. S3 Effects ofazole compounds on the activity and fluorescence of PHD3. (a) - (d) Inhibition of IPA (a), TPA (b), BIPA (c) and TEB (d) on the hydroxylation activity of PHD3. Incubations were carried out by mixing all cofactors and substrates, together with increasing amount of inhibitors (0-100 μM). IC_{50} values were estimated by dose regression of initial velocity versus concentration of inhibitors. The data was analyzed as mean ±S.D. of three independent experiments. (e) - (h) Fluorescence quenching of IPA (e), TPA (f), BIPA (g) and TEB (h) on PHD3-Fe^{2+} complex. The fluorescence emission spectra were taken in 100 mM PBS (pH 7.0) at 37 °C. 5 μM PHD3 and 50 μM Fe^{2+} were pre-incubated on ice. The maximum λ (emission) was quenched with increasing amounts of compounds (0-100 μM) at the excitation wavelength of 280 nm. Inset: Hill plots of the fluorescence quenching data in the maximum emission wavelength. The K_{d} values were estimated by fitting the Hill equation.
Fig. S4 Fluorescence quenching of BIPA (a), BTPA (b), TEB (c) and BEBT (d) on PHD3-Fe$^{2+}$-NOG complex. The fluorescence emission spectra were taken in 100 mM PBS (pH7.0) at 37 °C. 5 μM PHD3, 50 μM Fe$^{2+}$ and 50 μM NOG were pre-incubated on ice. The maximum λ (emission) was quenched with increasing amounts of compounds (0-100 μM) at the excitation wavelength of 280 nm. Inset: Hill plots of the fluorescence quenching data in the maximum emission wavelength. The $K_d$ values were estimated by fitting the Hill equation.
Fig. S5 Comparison of the CD spectra of PHD3-Fe (a) and PHD3-Fe-NOG (b) mixtures without (solid line) and with 50 μM IPA (dashed line), TPA (dotted line), BIPA (dash dotted line), BTPA (dash dot dotted line), TEB (short dashed line) and BEBT (short dash dotted line).
4. Supplementary References