Supporting Information

1,3,8-Triazaspiro[4.5]decane-2,4-diones as Efficacious Pan-inhibitors of Hypoxia-Inducible Factor Prolyl Hydroxylase 1-3 (HIF PHD1-3) for the Treatment of Anemia

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1. Chemistry

1a) Chemistry, General experimental procedures: Unless specified otherwise, all materials were purchased form commercial sources and used as received. ^{1}H and ^{13}C NMR spectra were recorded using a Brooker 400MHz instruments. Preparative reverse phase liquid chromatography (RPHPLC) was performed using Waters MS Directed Purification System consisting of 2525 Binary Gradient Pump, 2767 Injector/Collector and 2996 PDA UV detector, mobile phase: gradient of water and acetonitrile (each cont. 0.1% TFA), column: Waters Xterra (50x3mm, 3.5 micron packing material). The purity of **1-11** has been determined by HPLC analysis (Electrospray Positive Ionization, Micromass ZQ single quadrupole)[1] as ≥ 95%. Preparation of **3ff** (oxindole → **1a** → **2ff** → **3ff**; Scheme in Table 1) represents a typical procedure used for the synthesis of spiroindoles **3a-3kk**. Preparation of **6c** (**8** → **9** → **10c** → **6c**; Scheme 1) represents a typical procedure used for the synthesis of spiroindoles **4f**, **4g**, **6a-rr**, **11a-11m**.

1a (R^3 = H): Oxindole (44 g, 330 mmol) was dissolved in diemthoxyethane (DME, 880 mL), and the solution was cooled to -78ºC. A solution of LiHMDS/DME (550 mL, prepared by dissolving 246g of 97% of LiHMDS in 750 mL of DME) was added over 40 min maintaining an internal temperature of < -55ºC. The suspension was warmed to -30ºC over 30 min and a solution of *N*-Boc-bis(2-chloroethyl)amine[2] (92.1 g, 380mmol) in DME (120 mL) was added. The reaction mixture was allowed to warm to room temperature and stirred 18h. An additional 280 mL of the LiHMDS solution was added in portions over 2 days. The reaction mixture was poured into 1.7 L of 2N HCl and ice and aged for 1h.

The mixture was diluted with 2L of ether/hexanes (1/1) and the layers separated. The organic layer was washed with water (1L), saturated sodium bicarbonate solution (1.5L), and brine (0.5L). The organic layer was dried over $MgSO_4$ and treated with Darco G-60. The mixture was filtered through $MgSO_4$ and the filtrate was concentrated to slurry. The slurry was filtered and the cake washed with hexanes affording 80.5g (81%) of **1a** as a white solid; LCMS: 2.4min, *m/z* $(M\text{-}BocH)^+ = 203.2$.

2ff (R^3 = H; R^2 = 4-biphenyl): In an oven-dried flask, **1a** (2.8g, 9.26 mmol) and 4-iodobiphenyl (3.37 g, 12.04 mmol) were dissolved in acetonitrile (100 ml), and the mixture was degassed with stream of nitrogen through the solution at 40°C for 20 minutes. Anhydrous potassium carbonate (3.84 g, 27.8 mmol), copper(I) iodide (0.441 g, 2.315 mmol), and *N,N'*-dimethylethylenediamine (0.204 g, 2.315 mmol) were added sequentially and the resulting reaction mixture was heated to 80°C for 15h under nitrogen and then cooled to room temperature. The crude mixture was diluted with ethyl acetate and washed with 0.1M HCl solution, dried over sodium sulfate, filtered and concentrated. Further purification of the desired product was accomplished by column chromatography on silica gel: eluting with a gradient of ethyl acetate in hexanes: 0-40%/1.3L yielding 3.1g (74%) of **2ff** as a white solid; LCMS: 4.10min, *m/z* $(M\text{-}BocH)^+ = 355.1$.

3ff: 4M hydrogen chloride solution in dioxane (60 mL, 240 mmol) was added to **2ff** (3g, 6.60 mmol) via syringe in one portion and the resulting mixture was stirred at room temperature for 1h, concentrated to an approximate volume of 30 mL and cooled to 10°C. Solids were collected by filtration, rinsed with 10mL of dioxane and dried in desiccator to yield deprotected amine intermediate as a white solid (6.0 mmol); LCMS 2.92min, *m/z* $(MH)^+ = 355.1$. To a solution of the amine intermediate (6.0 mmol) in methylene chloride (120mL), 3-methylpyridine-2-carboxaldehyde (6.6 mmol) and sodium triacetoxy-borohydride (18 mmol) were added sequentially and the resulting mixture was stirred at ambient temperature for 2h. Methanol (60mL) was added and the resulting mixture stirred at room temperature for 5 minutes and concentrated. The final purification was accomplished by preparative reverse phase HPLC to yield 2.9g (77%) of **3ff** a white trifluoroacetic acid salt; 1H NMR (500MHz, CD3OD): 2.32 (m, 2H), 2.41 (s, 3H), 2.57 (m, 2H), 3.75 (m, 2H), 4.09 (m, 2H), 4.70 (s, 2H), 6.95 (d, J=8.2Hz, 1H), 7.22 (t, J=5.5Hz, 1H), 7.34 (t, J=5.5Hz, 1H), 7.38 (m, 1H), 7.54 (m, 2H), 7.63 (d, J=9.2Hz, 2H), 7.74 (d, J=9.6Hz, 2H), 7.85 (d, J=9.2Hz, 2H), 7.90 (d, J=7.8Hz, 1H), 8.18 (d, J=7.8Hz, 2H), 8.78 (d, J=3.2Hz, 1H); LCMS: 3.15min, m/z (MH)+ = 460.1.

9: A solution of **8**[3] (23.8 mmol), 4-iodobiphenyl (22.6 mmol) in acetonitrile (80 mL) and DMF (80 mL) was degassed with a stream of nitrogen for 15 min. *N,N'*-dimethylethylenediamine (5.94 mmol), copper(I) iodide (5.94 mmol), and potassium carbonate (71.3 mmol) were added sequentially. The resulting mixture was heated to 85°C for 15h, cooled to room temperature and combined with ethyl acetate (300 mL) and water (300 mL). The organic layer was separated and washed with water (2 x 300 mL), dried over sodium sulfate and concentrated. Purification by column chromatography on silica gel, eluted with a gradient of 0-100% hexanes in ethyl acetate, yielded 8.3g (83%) of **9c** as a white solid; LCMS: 3.21min, *m/z* $(MH\text{-}Boc)^+ = 321.0$.

10c (Ar = 4-methoxypyrimidine-2-yl): A solution of **9** (10 mmol), 2-iodo-4-methoxypyrimidine (30 mmol) in acetonitrile (20 mL) and DMF (20 mL) was degassed with a stream of nitrogen for 15 minutes. 2,2,6,6-tetramethyl-3,5-heptadion (10 mmol), copper(I) iodide (10 mmol), and cesium carbonate (50 mmol) were added sequentially and the resulting mixture was heated to 85°C for 15h and allowed to cool to room temperature. The mixture was combined with ethyl acetate (300 mL) and water (300 mL). The organic layer was separated and washed with water (2 x 300mL), dried over sodium sulfate and concentrated. Purification by preparative HPLC yielded 5.0g (78%) of **10c** as a white salt of triflouroacetaic acid; LCMS: 4.21min, *m/z* $(MH\text{-}Boc)^+ = 430.2$.

6c; *Boc-deprotection*: 4M solution of hydrogen chloride in dioxane (60 ml, 240 mmol) was added to **10c** (6 mmol) via syringe. The resulting mixture was stirred at room temperature for 1h, concentrated to an approximate volume of 30 mL and cooled to 10°C. Deprotected amine was isolated as a white solid by filtration, rinsed with 10mL of dioxane and dried in desiccator and used in the subsequent step without purification.

Reductive amination: The deprotected amine (4 mmol) was dissolved in methylene chloride (40mL). 3-Methylpyridine-2-carboxaldehyde (5 mmol) and sodium triacetoxyborohydride (20 mmol) were added to the solution sequentially. The resulting mixture was stirred at room temperature for 2h. Methanol (20mL) was added and the resulting mixture was stirred at ambient temperature for 5 min and then concentrated. The purification was accomplished by preparative reverse phase HPLC yielding 2.4g (93%) of *O*-methyl-**6c** as a white salt of trifluoroacetic acid; ^{1}HNMR ($CDCl_3$): δ = 2.03-2.15 (m, 4H), 2.03 (s, 3H), 3.53-4.30 (m, 6H), 4.06 (s, 3H), 6.60-8.49 (m, 14H); LCMS: 1.71min, *m/z* $(MH)^+$ = 535.

Demethylation: The *O*-methyl-**6c** (3.6 mmol) was combined with 1M aqueous solution of hydrochloric acid (20 mL), the resulting mixture was refluxed in a sealed tube for 8h and concentrated. The final purification was accomplished by preparative reverse phase HPLC yielding 2.0g (89%) of **6c** as a white salt of trifluoroacetic acid; ^{1}HNMR(CDCl3): δ = 1.96 (s, 3H), 2.24 (m, 2H), 2.34 (m, 2H), 3.84 (m,2H), 3.92 (m,2H), 4.48 (s, 2H), 6.18 (d,1H, J=6.6Hz), 7.24-7.87 (m, 12H), 8.43 (d,1H, J= 4.3 Hz); LCMS: 1.60min, *m/z* (MH)+ = 521.

3p: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 9.02 (d, *J* = 2.1 Hz, 1H), 8.62 (s, 1H), 8.17 (d, *J* = 8.5 Hz, 1H), 8.10 (d, *J* = 8.2 Hz, 1H), 7.92 (t, *J* = 8.0 Hz, 1H), 7.77 (t, *J* = 7.8 Hz, 1H), 7.61 (m, 1H), 7.56 (d, J=6.8Hz, 1H), 7.52 (s, 1H), 7.33 (t, *J* = 7.8 Hz, 1H), 7.24 (t, *J* = 7.5 Hz, 1H), 6.95 (d, *J* = 8.0 Hz, 1H), 5.21 (s, 2H), 4.17 (s, 2H), 3.20 (m, 2H), 2.96 (m, 2H), 2.16 (m, 4H); LCMS: 1.35 min, *m/z* $(MH)^+$ = 468.0.

3v: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 9.24 (d, *J* = 2.3 Hz, 1H), 8.97 (d, *J* = 2.0 Hz, 1H), 8.54 (d, *J* = 4.6 Hz, 1H), 8.24 (d, *J* = 8.7 Hz, 1H), 8.20 (d, *J* = 8.2 Hz, 1H), 8.03 (t, *J* = 7.1 Hz, 1H), 7.86 (t, *J* = 7.8 Hz, 1H), 7.74 (d, *J* = 7.6 Hz, 1H), 7.60 (d, *J* = 7.3 Hz, 1H), 7.38 (m, 2H), 7.30 (t, *J* = 7.5 Hz, 1H), 7.07 (d, *J* = 7.8 Hz, 1H), 4.72 (s, 2H), 4.08 (m, 2H), 3.78 (m, 2H), 2.65 (m, 2H), 2.41 (s, 3H), 2.37 (m, 2H); LCMS: 1.54 min, *m/z* $(MH)^+$ = 435.0.

3w: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (500MHz, CD3OD): 0.77 (m, 2H), 1.1 (m, 2H), 1.99 (m, 1H), 2.38 (m, 2H), 2.59 (m, 2H), 3.77 (m, 2H), 4.10 (m, 2H), 4.88 (s, 2H), 6.98 (d, J=7.8Hz, 1H), 7.27 (t, J=7.6Hz, 1H), 7.38 (m, 2H), 7.58 (m, 2H), 7.76 (t, J=7.1Hz, 1H), 7.91 (t, J=7.1Hz, 1H), 8.53 (d, J=3.5Hz, 1H), 8.59 (s, 1H), 9.00 (s, 1H); LCMS: 2.80min, m/z (MH)+ = 461.0.

3bb: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 8.47 (d, *J* = 2.5 Hz, 1H), 7.94 (dd, *J* = 8.7, 2.7 Hz, 1H), 7.72 (d, *J* = 8.9 Hz, 1H), 7.62 (m, 2H), 7.59 (d, *J* = 1.6 Hz, 1H), 7.54 (d, *J* = 1.6 Hz, 1H), 7.51 (d, *J* = 7.3 Hz, 1H), 7.29 (t, *J* = 7.6 Hz, 1H), 7.18 (t, *J* = 7.5 Hz, 1H), 6.86 (d, *J* = 7.8 Hz, 1H), 6.33 (m, 2H), 4.40 (s, 2H), 3.99 (s, 3H), 3.45 (m, 2H), 3.19 (m, 2H), 2.25 (m, 2H), 2.14 (m, 2H); LCMS: 1.42 min, *m/z* $(MH)^+$ = 439.2.

3cc: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 7.57 (d, *J* = 1.6 Hz, 1H), 7.52 (d, *J* = 1.4 Hz, 1H), 7.48 (d, *J* = 7.6 Hz, 1H), 7.32 (d, *J* = 8.0 Hz, 1H), 7.23 (t, *J* = 7.8 Hz, 1H), 7.15 (s, 1H), 7.12 (d, *J* = 7.6 Hz, 1H), 7.09 (dd, *J* = 7.8, 2.0 Hz, 1H), 6.73 (d, *J* = 7.8 Hz, 1H), 4.30 (s, 2H), 3.98 (s, 3H), 3.34 (m, 2H), 3.10 (m, 2H), 2.33 (d, *J* = 6.4 Hz, 1H), 2.5 (m, 4H); LCMS (Method A): 1.61 min, *m/z* $(MH)^+$ = 401.0.

3dd: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 7.47 (d, *J* = 7.5 Hz, 1H), 7.37 (m, 4H), 7.26 (t, *J* = 7.8 Hz, 1H), 7.15 (m, 4H), 7.07 (d, *J* = 8.3 Hz, 1H), 6.92 (s, 1H), 6.78 (d, *J* = 8.0 Hz, 1H), 4.51 (s, 2H), 4.00 (s, 3H), 3.55 (m, 2H), 3.30 (m, 2H), 2.28 (m, 2H), 2.12 (m, 2H); LCMS (Method A): 1.72 min, *m/z* $(MH)^+$ = 465.3.

3ee: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 7.83 (d, *J* = 8.4 Hz, 1H), 7.69 (d, *J* = 7.6 Hz, 1H), 7.54 (s, 2H), 7.49 (m, 5H), 7.38 (t, *J* = 7.1 Hz, 1H), 7.28 (t, *J* = 7.6 Hz,

1H), 7.17 (t, *J* = 7.6 Hz, 1H), 6.87 (d, *J* = 7.8 Hz, 1H), 4.18 (m, 2H), 3.97 (s, 3H), 2.25 (m, 2H), 3.00 (m, 2H), 2.12 (m, 4H); LCMS: 1.75 min, *m/z* $(MH)^+$ = 449.3.

3gg: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 9.23 (s, 1H), 8.88 (d, *J* = 8.0 Hz, 1H), 8.85 (d, *J* = 5.0 Hz, 1H), 8.55 (d, *J* = 4.6 Hz, 1H), 8.12 (dd, *J* = 8.0, 5.7 Hz, 1H), 8.04 (d, *J* = 8.7 Hz, 2H), 7.75 (d, *J* = 6.9 Hz, 1H), 7.73 (d, *J* = 8.4 Hz, 2H), 7.57 (d, *J* = 7.8 Hz, 1H), 7.37 (m, 2H), 7.26 (t, *J* = 7.6 Hz, 1H), 6.96 (d, *J* = 7.8 Hz, 1H), 4.72 (s, 2H), 4.08 (m, 2H), 3.76 (m, 2H), 2.60 (m, 2H), 2.41 (s, 3H), 2.32 (m, 2H); LCMS: 2.15 min, *m/z* $(MH)^+$ = 461.3.

3hh: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (400 MHz, CD_3OD) δ 8.79 (d, *J* = 5.2, 1H), 8.53 (d, *J* = 4.8, 1H), 8.35 (t, *J* = 7.8, 1H), 8.22 (d, *J* = 8.0, 1H), 8.17 (d, *J*= 8.5, 2H), 7.75 (m, 4H), 7.55 (d, *J* = 7.1, 1H), 7.36 (m, 2H), 7.25 (t, *J* = 7.6, 1H), 6.97 (d, *J* = 7.7, 1H), 4.71 (s, 2H), 4.06 (dt, *J* = 3.0, 12.3, 2H), 3.74 (d, *J* = 9.2, 2H), 2.59 (t, *J* = 11.8, 2H), 2.39 (s, 3H), 2.29 (d, *J* = 15.4, 2H). LCMS: 1.41 min, *m/z* $(MH)^+$ = 461.0.

3ii: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (500MHz, CD3OD): 2.33 (m, 2H), 2.39 (s, 3H), 2.56 (m, 2H), 3.75 (m, 2H), 4.09 (m, 2H), 4.70 (s, 2H), 6.94 (d, J=8.0Hz, 1H), 7.70 (m, 2H), 7.85-7.95 (m, 6H), 8.21 (m, 3H), 8.40 (m, 2H), 8.78 (d, J=3.2Hz, 1H); LCMS: 3.25min, m/z (MH)+ = 537.1/539.1.

3jj: The final purification was accomplished by preparative reverse phase HPLC to give the title compound, isolated as a salt of trifluoroacetic acid; ^{1}H NMR (CDCl3, 500MHz) δ 8.56 (d, J = 3.7 Hz, 1H), 7.94 (s, 1H), 7.87 (d, J = 8.4 Hz, 2H), 7.76 (t, J = 7.3 Hz, 2H), 7.71 (d, J = 7.3 Hz, 2H), 7.56 (d, J = 8.2 Hz, 2H), 7.52 (t, J = 7.6 Hz, 2H), 7.41 (m, 2H), 7.05 (d, J = 8.2 Hz, 1H), 4.75 (s, 2H), 4.07 (m, 2H), 3.75 (m, 2H), 2.58 (m, 2H), 2.41 (s, 3H), 2.38 (m, 2H); LCMS: 3.17min, m/z (MH)+ = 485.4.

11l was prepared according to the general procedure described for **6c** (using 4-iodopyrimidine as the ArI) and isolated as a white solid of trifluoroacetic acid; **11l**: ^{1}HNMR(CD3OD): δ = 2.42 (s, 3H), 2.45 (m, 2H), 3.80 (m, 2H), 3.80-3.94 (m, 2H), 4.04-4.10 (m, 2H), 4.76 (s, 2H), 7.37 (m, 1H), 7.64 (d, J= 8.5Hz, 2H), 7.74 (d, J = 7.6 Hz, 1H), 7.79 (d, J=8.5Hz, 2H), 7.86 (d, J= 8.5 Hz, 2H), 8.14 (d, J= 8.3 Hz, 2H), 8.46 (m, 1H), 8.54 (d, J=4.7Hz, 1H), 8.74 (d, J = 5.9 Hz, 1H), 9.06 (s, 1H); LCMS: 1.54 min, *m/z* $(MH)^+$ = 549.3.

<u>1b) HTE optimization of reaction conditions for C-N coupling</u> on spirohydantoin core (Scheme 1; step c: **9** → **10**); Screen performed in 96well plate with glass inserts, sealed with Teflon septum. General screen conditions: 0.5 euivalents of CuI vs. **9**, ligand/CuI ratio = 3.5/1, DMF/MeCN = 1/1 and heated to 110°C, conversion monirored by LCMS against an internal standard after 20h.

Ligand	DMEDA[a]		DACH[b]		proline		*N,N*-diMe-glycine		TMHD[c]		1,10-phenantroline	
ArX/Base B	B1[d]	B2[e]	B1[d]	B2[e]	B1[d]	B2[e]	B1[d]	B2[e]	B1[d]	B2[e]	B1[d]	B2[e]
PhI	32	24	1	5	0	5	1	46	57	98	2	3
2-iodopyridine	33	95	9	65	1	13	1	78	45	100	64	100
3-iodopyridine	3	5	1	0	1	6	1	1	19	71	2	2
2-I-pyridine-4-CO_2H	1	24	0	1	0	0	0	1	1	94	0	73
2-bromopyrimidine	0	0	0	0	0	1	0	0	2	47	4	41
2-iodothiophene	0	3	0	0	0	2	0	17	2	36	0	3

3-iodothiophene	2	33	0	0	0	6	0	56	20	90	2	11
Control (no ArX)	0	0	0	0	0	0	0	0	0	0	0	0

a: dimethylethylenediamine; b: 1,2-diaminocyclohexane (trans, racemic); c: 1,1,6,6-tetramethyl-3,5-heptadione; d: B1 = K_3PO_4; e: B2 = Cs_2CO_3.

2. In vitro assay for HIF-PHD2 catalytic activity represents a general procedure used for the determination of HIF-PHD catalytic activity for each of the three subtypes, HIF-PHD1-3: To each well of a 96-well plate was added 1 µL of test compound in DMSO and 20 µl of assay buffer (50 mM Tris pH 7.4/0.01% Tween-20/0.1 mg/ml bovine serum albumin/10 µM ferrous sulfate/1 mM sodium ascorbate/20 µg/ml catalase) containing 0.15 µg/ml FLAG-tagged full length PHD2 expressed in and purified from baculovirus-infected Sf9 cells. After a 30 min preincubation at room temperature, the enzymatic reactions were initiated by the addition of 4 µL of substrates (final concentrations of 0.2 µM 2-oxoglutarate and 0.5 µM HIF-1α peptide biotinyl-DLDLEMLAPYIPMDDDFQL). After 2 hr at room temperature, the reactions were terminated and signals were developed by the addition of a 25 µL quench/detection mix to a final concentration of 1 mM ortho-phenanthroline, 0.1 mM EDTA, 0.5 nM anti-$(His)_6$ LANCE reagent (Perkin-Elmer Life Sciences), 100 nM AF647-labeled streptavidin (Invitrogen), and 2 µg/ml $(His)_6$-VHL complex (S. Tan (2001) Protein Expr. Purif. 21, 224-234). The ratio of time resolved fluorescence signals at 665 and 620 nm was determined, and percent inhibition was calculated relative to an uninhibited control sample run in parallel.

3. Ligand-binding AS-MS protocol:

For the primary screen, 400,000 compounds were divided into mixtures of 205 compounds. For hit confirmation, mixtures of 5 compounds were combined with a final screening concentration of 5 µM per compound. A 2x solution of each ligand mixture with a final screening concentration of 2 µM per mixture component in DMSO was diluted in binding buffer (50 mM Tris, pH 7.5, 25 uM $FeCl_2$, 0.5 mM Ascorbate) with 10% DMSO (5% DMSO final), and centrifuged for 10 min at 17933 x g at 25 ^oC. An equal volume of the 2x ligand solution was added to a 2x solution of Flag-PHD2 (10 µM final protein concentration). The samples were incubated at 25 ^oC for 30 min followed by centrifugation at 1266 x g for 30 min at 4 ^oC. Samples were then transferred to a 4 ^oC autosampler of an Agilent 1100 Series HPLC. AS/MS analysis was performed on an integrated SEC-LC-MS platform. 1.5 µl of each sample was injected into a mobile phase of NaH_2PO_4, pH 7.5, at a 300 µl/min flow rate for fast SEC separation with a polyhydroxyethyl aspartamide column (Poly LC, 2.1 x 50 mm, 5 µm particle size, 60 Å pore size, The Nest Group). UV-based detection triggered a valve to trap and divert the excluded protein peak to the C18 analytical RPC (0.5 x 50 mm, 5 µm particles size, 100 Å pore size, Higgens Analytical) at a flow rate of 10 µl/min. Small molecules were dissociated from the protein with 5% CH_3CN/ 95% H_2O in 0.2% formic acid for 3 min, then, under standard conditions, separated with a gradient of 50% CH_3CN/ 50% H_2O, 0.2% formic acid to 95% CH_3CN/ 5% H_2O, 0.2% formic acid over 4 min. The samples flowed directly into the LCT Premiere electrospray time-of-flight mass spectrometer (Waters). Data was analyzed with either MassLynx (Waters) or a custom software package to identify hits.

4. On- and off-traget in vivo assays:

4a) In Vivo Evaluation Sequence

- Primary in vivo screen - MoPED (EPO 4 hr post IV dose)
- Secondary in vivo screen - reticulocytes (days 3 & 4 post PO dose)
- Hepatotoxicity screen - MALTED (IV ALT induction)
- PK/PD Analyses - single dose - dose-response & time-course, EPO/PK
- Efficacy Models - multiple dose - dose-response & time-course, hematology
- Disease Models - anemia model (5/6 nephrectomy)

- Pulmonary hypertension models - multiple dose with MRI, single dose with PAP telemetry

4b) General Analytical Methods

Epo/VEGF; ELISA: Measurements are performed using serum or plasma with the MesoScale Discovery Mouse/Rat Hypoxia Serum/Plasma Kit, cat# K11123C as per the manufacturer's instructions.

Reticulocytes; FACS: Reticulocyte Analysis - Thiazole Orange

Materials/reagents: Thiazole Orange Powder (Polysciences, #19352); Polybead® Polystyrene microspheres; 2.0 microns (Polysciences, #19814); Methanol; Dulbecco's Phosphate Buffered Saline; Polystyrene round-bottom tubes, 12 x 75 mm (Falcon®, #2058); Thiazole Orange Powder -store at room temperature; protect from light; Thiazole Orange Stock Solution - dissolve 10 mg thiazole powder in 10 ml methanol, store in the dark at -20˚C. (stable for 2 months); Thiazole Orange Working Solution - combine 10 µl of stock solution with 100 ml of DPBS, add 10 µl of Polybead® microspheres, prepare fresh daily; store in brown bottle to protect from light.

Procedure: Collect blood into EDTA tubes; Label appropriate number of Falcon tubes, including appropriate controls (unstained sample, beads only sample). To unstained sample, add 2 ml of DPBS. To all 'bead only' and 'test' sample tubes add 2 ml of Thiazole Orange working solution. Add 3 µl of blood to each "test' tube (for unstained sample use vehicle control blood). Gently vortex thoroughly to mix.. Incubate at room temperature for 30 minutes, protected from light. FACS - collect 100,000 events on BD FACSCalibur for FSC, SSC and FL1. Note and record flow setting. Analyze using FlowJo. Gate total RBCs using FSC X SSC, quantitate FL1 positive RBCs using FL1 histogram. Report results as % of RBCs, total reticulocytes and total RBCs.

ALT (hepatotoxicity indicator); Male C57Bl/6 mice (~8 weeks old); n=3 to 5. Formulations: soluble aqueous including cosolvents hydroxypropyl-□-cyclodextrin or PEG200, also EtOH or DMSO and as needed within recommended safety limits. 0 hr: Vehicle or compound, IV, IP and or PO dosed.; 4 hr or 6 hr or 24 hr post dose - blood collection via cardiac puncture at time of CO_2 euthanasia, serum collected after clotting. Reagents: ALT (SGPT) SUBSTRATE: 0.2 M L-alanine, 2.0 mM α-ketoglutarate, 100 mM phosphate buffer at pH 7.4. ALT (SGPT) COLOR REAGENT: 1.0 mM 2, 4-dinitrophenylhydrazine in 1N hydrochloric acid. ALT (SGPT) Color Developer: 0.5N sodium hydroxide.ALT (SGPT) CALIBRATOR STOCK: Solution of 10mM sodium pyruvate in 100 mM phosphate buffer at pH 7.4. Procedure for Serum ALT Assay: Alanine aminotransferase (ALT) (SGPT) reagents were prepared in-house (compositions above based on a commercial kit from Teco Diagnostics, Anaheim, CA). The assay method was scaled from the Teco protocol for use in 96-well flat-bottomed microplates as follows. A volume of 50 µl of ALT substrate was placed in each well of a 96-well plate, and 10 µl of neat or diluted (1:10) sample/standard was added at timed intervals. The samples were incubated for 30 min at 37°C, after which 50 µl of ALT color reagent was added to each sample and incubated for 10 min as above. A volume of 200 µl of ALT color developer was then added to each well and incubated for 5 min at 37°C. The plate was then read at 505 nm on a spectrophotometer and the ALT concentration determined by interpolation from a standard curve, sodium pyruvate dilution series in PBS of 10, 5, 2.5, 1.25, 0.625, 0.3125, 0.15, and 0 mM in duplicate on the microtiter plate containing the unknowns. Results were analyzed using a non-linear fit (KaleidaGraph, version 3.52) of the pyruvate standard curve [equation Y=A/(1+B/X)+C]. Unknowns were interpolated from the fit parameters. Normal expected values in mouse range from 5-35 U/L.

MRI (pulmonary hypertension). Measurement of ventricular ejection fraction and ventricular wall thickness (alternative to histological evaluation of pulmonary hypertension). Procedure performed by Imaging Group, Rahway (Center of Excellence); Formulation: Cyclodextrin - compound is dissolved in ½ final volume of stock solution (40% w/v, Sigma # 332607) plus pH adjuster in water (typically 1 equivalent of NaOH) using heat and sonication. Water is then added to final volume. PEG/water (polyethylene glycol 200 / water 25/75, various commercial sources) - compound is dissolved in ½ final volume of PEG/water 1:1 plus pH adjuster in water (typically 1 equivalent of NaOH) using heat and sonication. Water is then added to final volume.

MoPED (Mouse Plasma Erythropoietin Determination): Compounds are formulated in cyclodextrin. Mice (C57Bl/6, n=3) are dosed IV in a volume of 0.2 ml. After 4 hours, blood is obtained via cardiac puncture upon euthanasia with CO2. Plasmas are stored at -80° C. and are assayed the following day for Epo/VEGF. Results are compared to vehicle dosed controls and the assay is quality controlled by inclusion of a positive control compound, dosed at 15 mg/kg. Variation - mice are dosed orally and euthanized at 5 hours post dose.

Secondary in vivo screen - reticulocytes (days 3 & 4 post PO dose): Compounds are formulated in cyclodextrin. Mice (C57Bl/6, n=10) are dosed PO in a volume of 0.2 ml. On days 3 and 4 post-dose, blood is obtained from 5 mice via cardiac puncture upon euthanasia with CO2. Blood is analyzed for reticulocytes by FACS. Results are compared to vehicle dosed controls and the assay is quality controlled by inclusion of a positive control compound, dosed at 15 mg/kg.

Hepatotoxicity screen - MALTED (IV ALT induction); Complete details are in Appendix A. Five animals per group with a 6 hour time course is used for ad hoc studies. For screening, MoPED samples (n=3, 4 hours) are assayed for ALT.

PK/PD Analyses in Rats: Compounds are formulated in PEG/water. Rats (SD, n=5) are dosed PO at a rate of 2.5 ml/kg. The studies include three compound treated groups in three-fold intervals plus a vehicle control. Dose range is selected based on potency in mouse studies and is typically 5, 15 & 50 mg/kg or 1.5, 5 & 15 mg/kg. Blood samples are obtained by jugular venipuncture at 6, 24, 48, 72, and 96 hours post-dose. Plasma is prepared from these samples by centrifugation and stored at -80° C. Plasmas up to 72 hours are assayed for Epo/VEGF, blood from 72 and 96 hour samples are analyzed for reticulocytes by FACS and all plasmas are submitted to DMPK for compound level analysis (PK). Epo and reticuloctedata provides potency and duration of action information, the washout period is determined by PK analysis and combined analysis yields exposure targets for efficacy.

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[1] Mass Spectrometer: Micromass ZQ single quadrupole, Electrospray Positive Ionization, Full Scan mode (150-750amu in 0.5s); HPLC: Agilent 1100, Binary Pump; DAD UV detector: Hardware/software Waters/Micromass MassLynx 4.0; Column: Waters Xterra, 2.1 mm Width, 20 mm Length, 3.5 micron packing material; Runtime: 4min; Flow Rate: 1.0 ml /min.; Mobile Phase A = Water + 0.05% TFA, B = Acetonitrile + 0.05% TFA; Gradient: Time/%A/%B: 0.00/95/05, 3.00/2/98, 3.25/2/98, 3.26/95/5, 4.00/95/5.

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[3] (a) Sarges, R.; Schnur, R.C.; Belletire, J.L.; Peterson, M.J. Spirohydantoin aldose reductase inhibitors. *J. Med. Chem.* **1988**, *31*, 230-43; (b) Courtoison, J.C.; Coudert, P.; Couquelet, J.; Tronche, P.; Jonadet, M.; Bastide, P. Synthesis and pharmacological testing of some spirohydantoins: relation to conformation. *Farmaco, Edizione Sci.* **1988**, *43*, 153-60.