**Supplementary Information**

**Evaluation of Aspirin Metabolites as Inhibitors of Hypoxia Inducible Factor Hydroxylases**

![Chemical Structures]

**Figure S1.** Links between aspirin (2) metabolites (black arrows), potential 2 metabolites (dashed arrows) and benzoic acid (12) and hippuric acid (7) metabolites (blue arrows) reported to date in the literature. Metabolites originating from the benzoate (12) degradation via hydroxylation pathway (from the KEGG PATHWAY Database)\(^1\) of the human detoxification process are also represented (orange arrows).

**Synthesis**

Compounds 8, 10, 14-18, 20, 23-26 have been previously reported (see corresponding references below). Synthetic procedures have not been described for 15, 17 and 23. Different routes from the published ones were used for the preparation of 8, 10, 18, 20, 25 and 26. Only compound 14 has been fully characterised. Therefore we report here the synthetic procedure and characterisation data for the compounds that were employed.

**Materials and methods:**
Solvents were either dried solvents (Aldrich) or dried by passing under nitrogen pressure over an aluminate column. All reagents were used as obtained from commercial sources unless otherwise stated. Measurement of pH was carried out using Prolabo Rota pH 1-10 paper. Flash chromatography was performed using silica gel (0.125-0.25 mm, 60-120 mesh) as the stationery phase. Thin layer chromatography (TLC) was performed on aluminium plates pre-coated with silica gel (Merck silica gel 60 F254 1.05554), which were visualised by the quenching of UV fluorescence (using an irradiation wavelength $\lambda_{\text{max}} = 254\text{nm}$), and/or by staining with iodine or 10% ammonium molybdate in 2M sulphuric acid, followed by heating. Melting points (mp) were obtained using a Büchi 510 Cambridge Instruments Gallen III hot stage melting point apparatus. Proton magnetic resonance spectra ($^1\text{H NMR}$) were recorded on Brüker DPX 200 (200MHz), Brüker DPX 250 (250MHz), Brüker DPX 400 (400MHz), Brüker DQX 400 (400MHz), Brüker DRX500 (500MHz), and Brüker AMX500 (500MHz) spectrometers at ambient and variable temperature. Carbon magnetic resonance spectra ($^{13}\text{C NMR}$) were recorded on Brüker DPX 200 (50.3MHz), Brüker DPX 250 (62.9MHz), Brüker DPX 400 (100.6MHz), Brüker DQX 400 (100.6MHz), Brüker DRX500 (125.8MHz), and Brüker AMX500 (125.8MHz) spectrometers at ambient temperature. Chemical shifts ($\delta$) are quoted in parts per million (ppm) and are referenced to the residual solvent peak. High-resolution mass spectra were recorded on a VG Autospec spectrometer by chemical ionisation or on a Micromass LCT electrospray ionisation mass spectrometer operating at a resolution of 5000 full width half heights.

**Preparation of compounds 8, 10, 14-24 for the coupling with appropriate glycine ester:**

Compounds 8, 10, 14-24 were prepared in two steps from commercially available salicylates and with overall yields ranging from 30 to 80 %. Esters intermediates 32, 36-41, were synthesized using the 1-ethyl-3-(3’-dimethylaminopropyl)carbodiimide/l-hydroxybenzotriazole (EDCI/HOBt) coupling method. Thus, the hydroxybenzoic acids were coupled with glycine methyl ester hydrochloride by EDCI/HOBt (Method a) as shown in Schemes 1 and 2. The nitro derivative, 42, was obtained in 50% yield when the above peptide coupling reaction was mediated by dicyclohexylcarbodiimide (DCC) in ethyl acetate (Method b). Reduction of the nitro group of 42 with 10% Pd/C gave the amino derivative 43 (Scheme 3).

Use of DCC in dry THF gave the best obtained yields formation of the glycine derivatives with two aromatic hydroxyl groups 28-31 (Method b). Alternative methods employing glycine free base,$^5$ EDCI/HOBt, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOC), $N,N'$-dicarboxyldimidazole, in different solvents and temperatures gave no reaction or lower yields. Hydrolysis of the esters employed aqueous NaOH.
Scheme 1. Reagents and conditions: (i) Method a: H$_2$NCH$_2$COOMe.HCl, HOBt, EDCI, Et$_3$N, CH$_2$Cl$_2$ (ii) NaOH.

Scheme 2. Reagents and conditions: (i) Method a: H$_2$NCH$_2$COOMe.HCl, HOBt, EDCI, Et$_3$N, CH$_2$Cl$_2$ for 37-41. Method b: H$_2$NCH$_2$COOEt.HCl, DCC, Et$_3$N, anhydrous THF for 28-31; (ii) NaOH.

Scheme 3. Reagents and conditions: (i) Method b: H$_2$NCH$_2$COOMe.HCl, DCC, Et$_3$N, anhydrous ethyl acetate; (ii) 10%Pd/C, MeOH; (iii) NaOH.

General procedure for the amide coupling reactions:
Method a: To a solution of the glycine methyl ester hydrochloride (1 eq) and triethylamine (2 eq) in chloroform (5 ml per mmol), HOBt (1.2 eq), the acid (1 eq) and EDCI (1.2 eq) were subsequently added. The reaction mixture was allowed to stir overnight, washed with 1 M HCl solution, a solution of saturated NaHCO₃, and then brine, dried (MgSO₄) and evaporated in vacuo. The crude products were purified by column chromatography using hexane: ethyl acetate as eluent to afford 32 and 36-41 (Yields: 30-40%).

Method b: To a solution of glycine ethyl ester hydrochloride (1 eq), triethylamine (1.1 eq) and the corresponding acid (1 eq) in dry THF or ethyl acetate (1-1.5 ml per mmol) was added dropwise at 0°C 1,3-dicyclohexylcarbodiimide (1.1 eq) in dry THF/ethyl acetate (0.5 ml per mmol). The reaction mixture was stirred at room temperature (rt) overnight, filtered; the filtrate was then evaporated in vacuo, diluted with ethyl acetate, washed with 0.01 M HCl, water and brine. The organic layer was dried over MgSO₄ and the solvent evaporated in vacuo. The crude products were purified by flash chromatography using hexane: ethyl acetate as eluent to afford 28-31 and 42 (Yields: 50-80%).

General method for ester hydrolysis saponification:
A mixture of the corresponding ester and NaOH (1 M, 3 eq) was stirred at room temperature until TLC showed that the starting material disappeared (2-12 hours). The solution was washed with ethyl acetate. The aqueous layer was then acidified with 1 M HCl and extracted with ethyl acetate, dried (Na₂SO₄) and the solvent evaporated in vacuo to afford 8, 10, 14-24.

Analytical data: Methyl N-[(pyridin-2-yl)carbonyl]glycinate, 36. ¹H-NMR (400 MHz, DMSO-d₆): δ 3.78 (s, 3H), 4.27 (d, J = 5.5 Hz, 2H), 7.44 (ddd, J = 1.0, 5.0, 7.5 Hz, 1H), 7.84 (td, J = 1.5, 7.5 Hz, 1H), 8.18 (dt, J = 1.0, 7.5 Hz, 1H), 8.50 (br, 1H), 8.57 (ddd, J = 1.0, 1.5, 5.0 Hz, 1H); ¹³C-NMR (100.6 MHz, DMSO-d₆): δ 41.7, 52.8, 122.7, 126.9, 137.8, 148.7, 149.7, 165.1, 170.6. N-[(pyridin-2-yl)carbonyl]glycine, 18. Mp: 167-168 °C, lit. mp: 164-165 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ 3.90 (d, J = 5.5 Hz, 2H), 5.87 (br, 1H), 7.59-7.62 (m, 1H), 7.98-8.05 (m, 2H), 8.66-8.67 (m, 1H), 8.82 (t, J = 5.5 Hz, 1H); ¹³C-NMR (100.6 MHz, DMSO-d₆): δ 42.8, 122.6, 127.5, 138.7, 149.4, 150.5, 164.4, 172.0. Methyl N-[(3-hydroxypyridin-2-yl)carbonyl]glycinate, 32. ¹H-NMR (400 MHz, CDCl₃): δ 3.81 (s, 3H), 4.24 (d, J = 6.0 Hz, 2H), 7.27-7.37 (m, 2H), 8.08 (d, J = 4.0 Hz, 1H), 8.47 (bs, 1H), 11.77 (s, 1H); ¹³C-NMR (100.6 MHz, CDCl₃): δ 40.7, 52.5, 126.1, 128.9, 131.0, 139.8, 157.7, 169.0, 169.6; HRESMS (m/z): [M – H]⁻ calcd. for C₉H₁₁N₂O₄ 211.0719; found 211.0717. N-[(3-hydroxypyridin-2-yl)carbonyl]glycine, 10. White solid. Mp: 167-171 °C. ¹H-NMR (500 MHz, D₂O): δ 4.27 (s, 2H), 7.95 (dd, J = 5.0, 8.5 Hz, 1H), 8.10 (d, J = 8.5 Hz, 1H), 8.35 (d, J = 5.0 Hz, 1H); ¹³C-NMR (128.5 MHz, D₂O): δ 41.8, 127.7, 130.5, 134.8, 135.1, 156.9, 161.2, 172.8; HRESMS (m/z): [M – H]⁻ calcd. for C₉H₁₁N₂O₄ 195.0406; found 195.0411. Ethyl N-(2,3-dihydroxybenzoyl)glycinate, 28. White solid. Mp: 138-140 °C. ¹H-NMR (400 MHz, DMSO-d₆): δ 1.20 (t, J = 7.0 Hz, 3H); 4.03 (d, J = 5.5 Hz, 2H), 4.13 (q, J = 7.0 Hz, 2H); 6.72 (t, J = 7.5 Hz, 1H), 6.94 (d, J = 7.5 Hz, 1H), 7.30 (d, J = 7.5 Hz, 1H), 9.19 (t, J = 5.5, 1H), 9.33 (bs, 1H); 12.20 (bs, 1H); ¹³C-NMR (100.6 MHz, DMSO-d₆): δ 14.9, 41.9, 61.5, 115.7, 118.4, 119.1, 119.9, 147.05, 150.11, 170.4, 170.7; HRESMS
(m/z): [M – H] calcd. for C_{11}H_{12}NO_{5} 238.0715; found 238.0727. N-(2,3-dihydroxybenzoyl)glycine, 14. dope 202-204 °C \textsuperscript{1}H-NMR (400 MHz, DMSO-\textit{d}_{6}): \delta 3.95 (d, J = 6.0 Hz, 2H), 6.71 (t, J = 8.0 Hz, 1H), 6.93 (dd, J = 1.0, 8.0 Hz, 1H), 7.30 (dd, J = 1.0, 8.0 Hz, 1H), 9.12 (t, J = 6.0 Hz, 1H), 9.27 (bs, 1H), 12.32 (bs, 1H), 12.62 (bs, 1H); \textsuperscript{13}C-NMR (100.6 MHz, DMSO-\textit{d}_{6}): \delta 41.8, 115.8, 118.4, 119.0, 119.9, 147.1, 150.2, 170.6, 171.8; HRESMS (m/z): [M – H] calcd. for C_{9}H_{8}NO_{5} 210.0402; found 210.0400. Ethyl N-(2,4-dihydroxybenzoyl)glycinate, 29. White solid. Mp: 139-141 °C \textsuperscript{1}H-NMR (400 MHz, DMSO-\textit{d}_{6}) \delta 1.19 (t, J = 7.0 Hz, 3H); 3.99 (d, J = 6.0 Hz, 2H), 4.12 (q, J = 7.0 Hz, 2H); 6.26 (d, J = 2.5 Hz, 1H), 6.32 (dd, J = 2.5, 9.0 Hz, 1H), 7.68 (d, J = 9.0 Hz, 1H), 8.93 (t, J = 6.0 Hz, 1H), 10.19 (bs, 1H); 12.42 (bs, 1H); \textsuperscript{13}C-NMR (100.6 MHz, DMSO-\textit{d}_{6}): \delta 14.9, 41.8, 61.4, 103.6, 107.5, 108.2, 130.3, 162.9, 163.3, 170.2, 170.6; HRESMS (m/z): [M – H] calcd. for C_{11}H_{12}NO_{5} 238.0715; found 238.0727. N-(2,4-dihydroxybenzoyl)glycine, 15. Mp: 202-203 °C \textsuperscript{1}H-NMR (400 MHz, DMSO-\textit{d}_{6}) \delta 3.92 (d, J = 6.0 Hz, 2H), 6.25 (d, J = 2.5 Hz, 1H), 6.31 (dd, J = 2.5, 9.0 Hz, 1H), 7.69 (d, J = 9.0 Hz, 1H), 8.87 (t, J = 6.0 Hz, 1H), 10.11 (s, 1H), 12.51 (s, 1H), 12.62 (bs, 1H); \textsuperscript{13}C-NMR (100.6 MHz, DMSO-\textit{d}_{6}): \delta 41.7, 103.5, 107.5, 108.1, 130.3, 162.9, 163.2, 170.1, 172.0; HRESMS (m/z): [M – H] calcd. for C_{9}H_{8}NO_{5} 210.0402; found 210.0404. Ethyl N-(2,5-dihydroxybenzoyl)glycinate, 30. White solid. Mp: 156-159 °C \textsuperscript{1}H-NMR (400 MHz, DMSO-\textit{d}_{6}) \delta 1.20 (t, J = 7.0 Hz, 3H); 4.03 (d, J = 5.5 Hz, 2H), 4.12 (q, J = 7.0 Hz, 2H), 6.77 (d, J = 9.0 Hz, 1H), 6.86 (dd, J = 3.0, 9.0 Hz, 1H), 7.25 (d, J = 3.0 Hz, 1H), 9.05 (bs, 2H); 11.25 (s, 1H); \textsuperscript{13}C-NMR (100.6 MHz, DMSO-\textit{d}_{6}) \delta 14.1, 41.2, 60.6, 113.9, 115.9, 117.8, 121.4, 149.5, 151.6, 168.0, 169.7; HRESMS (m/z): [M – H]\textsuperscript{+} calcd. for C_{11}H_{12}NO_{5} 238.0710; found 238.0710. N-(2,5-dihydroxybenzoyl)glycine, 16.8 Slightly brown solid. Mp: 209-211 °C, lit. mp: 204-205, \textsuperscript{1}H-NMR (400 MHz, DMSO-\textit{d}_{6}) \delta 3.96 (d, J = 5.5 Hz, 2H), 6.75 (d, J = 9.0 Hz, 1H), 6.85 (dd, J = 3.0, 9.0 Hz, 1H), 7.25 (d, J = 3.0 Hz, 1H), 9.00 (t, J = 5.5 Hz, 1H), 9.04 (bs, 1H), 11.30 (s, 1H), 12.67 (bs, 1H); \textsuperscript{13}C-NMR (128.5 MHz, DMSO-\textit{d}_{6}) \delta 42.0, 114.7, 116.8, 118.7, 122.1, 150.3, 152.4, 168.8, 171.9; HRESMS (m/z): [M – H]\textsuperscript{+} calcd. for C_{9}H_{8}NO_{5} 210.0397; found 210.0404. Ethyl N-(2,6-dihydroxybenzoyl)glycinate, 31. White solid. Mp: 172-173 °C. \textsuperscript{1}H-NMR (400 MHz, DMSO-\textit{d}_{6}) \delta 1.21 (t, J = 7.0 Hz, 3H); 4.12 (d, J = 5.5 Hz, 2H), 4.14 (q, J = 7.0 Hz, 2H); 6.38 (d, J = 8.0 Hz, 2H), 7.19 (t, J = 8.0 Hz, 1H), 9.25 (t, J = 5.5 Hz, 1H), 12.49 (s, 2H); \textsuperscript{13}C-NMR (100.6 MHz, DMSO-\textit{d}_{6}) \delta 14.09, 41.2, 62.2, 102.3, 108.1 (x2), 133.5, 159.7 (x2), 170.8, 171.6; HRESMS (m/z): [M – H]\textsuperscript{+} calcd. for C_{9}H_{8}NO_{5} 238.0710; found 238.0719. N-(2,6-dihydroxybenzoyl)glycine, 17.4 Mp: 198-200 °C \textsuperscript{1}H-NMR (200 MHz, CD_{3}OD): \delta 4.19 (s, 2H), 6.41 (d, J = 8.0 Hz, 2H), 7.19 (t, J = 8.0, 1H); \textsuperscript{13}C-NMR (128.5 MHz, CD_{3}OD): \delta 42.1, 103.8, 108.4 (x2), 134.6, 161.9 (x2), 172.2, 172.9; HRESMS (m/z): [M – H]\textsuperscript{+} calcd. for C_{9}H_{8}NO_{5} 208.0615; found 208.0618. Methyl N-(2-hydroxybenzoyl)glycinate, 37. White solid. Mp: 59-61 °C. \textsuperscript{1}H-NMR (400 MHz, CDCl\textsubscript{3}) \delta 3.83 (s, 3H), 4.23 (d, J = 5.0 Hz, 2H), 6.87 (t, J = 7.5 Hz, 1H), 6.91 (bs, 1H), 6.98 (d, J = 8.5 Hz, 1H), 7.40-7.46 (m, 2H), 12.01 (s, 1H); \textsuperscript{13}C-NMR (100.6 MHz, DMSO-\textit{d}_{6}) \delta 41.3, 52.7, 113.6, 118.6, 118.8, 125.7, 134.6, 161.5, 170.0, 170.1. HRESMS (m/z): [M – H]\textsuperscript{+} calcd. for C_{9}H_{8}NO_{5} 208.0615; found 208.0618. N-(2-hydroxybenzoyl)glycine, 8.10 White solid. Mp: 164-165 °C. \textsuperscript{1}H-NMR (400 MHz, DMSO-\textit{d}_{6}) \delta 3.98 (d, J =
6.0 Hz, 2H), 6.91 (m, 2H), 7.41 (t, \( J = 8.0 \), 1H), 7.87 (d, \( J = 8.0 \) Hz, 1H), 9.13 (t, \( J = 6.0 \) Hz, 1H), 12.21 (s, 1H), 12.73 (bs, 1H); \(^{13}\)C-NMR (100.6 MHz, DMSO-\(d_6\)): \( \delta \) 41.1, 115.3, 117.4, 118.8, 128.3, 133.9, 159.5, 168.7, 171.0. Methyl \(-\text{(4-chloro-2-hydroxybenzoyl)glycinate, 38}\). White solid. Mp: 146-149 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \( \delta \) 3.84 (s, 3H), 4.22 (d, \( J = 5.0 \) Hz, 2H), 6.84 (dd, \( J = 2.0, 9.0 \) Hz, 1H), 6.93 (bs, 1H), 6.98 (d, \( J = 2.0 \) Hz, 1H), 7.36 (d, \( J = 9.0 \) Hz, 1H), 12.19 (bs, 1H); \(^{13}\)C-NMR (100.6 MHz, CDCl\(_3\)): \( \delta \) 41.3, 52.8, 112.1, 118.6, 119.3, 126.7, 140.2, 162.3, 169.4, 170.2; HRESMS (m/z): \([\text{M} + \text{Na}]^+\) calcd. for \( \text{C}_{10}\text{H}_{10}\text{CINaNO}_4 \) 266.0191; found 266.0190. N-(4-chloro-2-hydroxybenzoyl)glycine, 19.\(^{11}\) White solid. Mp: 204-206 °C; \(^1\)H-NMR (400 MHz, CD\(_3\)OD): \( \delta \) 4.12 (s, 2H), 6.92 (dd, \( J = 2.0, 9.0 \) Hz, 1H), 6.95 (d, \( J = 2.0 \) Hz, 1H), 7.83 (d, \( J = 9.0 \) Hz, 1H); \(^{13}\)C-NMR (100.6 MHz, CD\(_3\)OD): \( \delta \) 41.0, 114.9, 117.2, 119.5, 129.8, 139.2, 160.7, 169.0, 171.9; HRESMS (m/z): \([\text{M} + \text{Na}]^+\) calcd. for \( \text{C}_{11}\text{H}_{13}\text{NNaO}_4 \) 246.0737; found 246.0742.

\( \text{N-(5-chloro-2-hydroxybenzoyl)glycinate, 39}\). White solid. Mp: 144-149 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \( \delta \) 3.84 (s, 3H), 4.23 (d, \( J = 5.0 \) Hz, 2H), 6.92 (d, \( J = 9.0 \) Hz, 1H), 6.96 (bs, 1H), 7.34 (dd, \( J = 2.0, 9.0 \) Hz, 1H), 7.43 (d, \( J = 2.0 \) Hz, 1H), 11.91 (bs, 1H); \(^{13}\)C-NMR (100.6 MHz, CDCl\(_3\)): \( \delta \) 41.3, 52.8, 114.5, 120.1, 123.5, 125.4, 134.5, 160.0, 169.0, 170.1; HRESMS (m/z): \([\text{M} + \text{Na}]^+\) calcd. for \( \text{C}_{10}\text{H}_{11}\text{CINaO}_4 \) 252.0034; found 252.0031.

\( \text{N-(2-hydroxy-4-methylbenzoyl)glycinate, 40}\). White solid. Mp: 110-113 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \( \delta \) 2.32 (s, 3H), 3.82 (s, 3H), 4.21 (d, \( J = 5.0 \) Hz, 2H), 6.67 (d, \( J = 8.0 \) Hz, 1H), 6.78 (s, 1H), 6.90 (bs, 1H), 7.32 (d, \( J = 8.0 \) Hz, 1H), 11.77 (bs, 1H); \(^{13}\)C-NMR (100.6 MHz, CDCl\(_3\)): \( \delta \) 21.6, 41.2, 52.7, 111.0, 118.6, 120.5, 125.5, 145.7, 161.5, 170.0, 170.3; HRESMS (m/z): \([\text{M} + \text{Na}]^+\) calcd. for \( \text{C}_{11}\text{H}_{12}\text{NNaO}_4 \) 246.0773; found 246.0741. N-(2-hydroxy-4-methylbenzoyl)glycine, 21.\(^{11}\) White solid. Mp: 209-212 °C; \(^1\)H-NMR (400 MHz, CD\(_3\)OD): \( \delta \) 2.32 (s, 3H), 4.11 (s, 2H), 6.73 (m, 2H), 7.68 (d, \( J = 2.0 \) Hz, 1H); \(^{13}\)C-NMR (100.6 MHz, CD\(_3\)OD): \( \delta \) 20.5, 40.9, 113.1, 117.6, 120.2, 128.1, 145.2, 160.1, 170.1, 172.1; HRESMS (m/z): \([\text{M} + \text{Na}]^+\) calcd. for \( \text{C}_{10}\text{H}_{12}\text{NNaO}_4 \) 232.0580; found 232.0579. Methyl \(-\text{(2-hydroxy-5-methylbenzoyl)glycinate, 41}\). White solid. Mp: 104-106 °C; \(^1\)H-NMR (400 MHz, CDCl\(_3\)): \( \delta \) 2.23 (s, 3H), 3.83 (s, 3H), 4.22 (d, \( J = 5.0 \) Hz, 2H), 6.87 (d, \( J = 8.5 \) Hz, 1H), 6.96 (bs, 1H), 7.21 (m, 2H), 11.77 (bs, 1H); \(^{13}\)C-NMR (100.6 MHz, CDCl\(_3\)): \( \delta \) 20.5, 41.2, 52.7, 113.2, 118.3, 125.6, 128.0, 135.5, 159.3, 170.0, 170.3; HRESMS (m/z): \([\text{M} + \text{Na}]^+\) calcd. for \( \text{C}_{11}\text{H}_{12}\text{NNaO}_4 \) 246.0737; found 246.0742. N-(2-hydroxy-5-methylbenzoyl)glycine, 22.\(^{11}\) White solid. Mp: 192-195 °C; \(^1\)H-NMR (400 MHz, CD\(_3\)OD): \( \delta \) 2.29 (s, 3H), 4.12 (s, 2H), 6.81 (d, \( J = 8.0 \) Hz, 1H), 7.21 (dd, \( J = 2.0, 8.0 \) Hz, 1H), 7.62 (d, \( J = 2.0 \) Hz, 1H); \(^{13}\)C-NMR (100.6 MHz, CD\(_3\)OD): \( \delta \) 19.5, 40.9, 115.4, 117.2, 128.2, 128.5, 134.7, 157.7, 170.0, 170.1; HRESMS (m/z): \([\text{M} + \text{Na}]^+\) calcd. for \( \text{C}_{10}\text{H}_{12}\text{NNaO}_4 \) 232.0580; found 232.0586. Methyl \(-\text{(2-hydroxy-5-nitrobenzoyl)glycinate 42}\). M.p. 145-148 °C; \(^1\)H NMR (400 MHz, DMSO-\(d_6\)): \( \delta \) 11.55 (brs, 1H), 8.96 (t, \( J = 5.0 \) Hz, 1H), 8.81 (d, \( J = 2.5 \) Hz, 1H), 8.25 (dd, \( J = 2.5, 9.0 \) Hz, 1H), 7.10 (d, \( J = 9.0 \) Hz, 1H).
Hz, 1H), 4.01 (d, J = 5.0 Hz, 2H), 3.81 ppm (s, 3H). N-(2-hydroxy-5-nitrobenzoyl)glycine, 23.13 Yellow solid. Mp: 178-180 °C. 1H-NMR (400 MHz, DMSO-d6): δ 4.03 (d, J = 5.0 Hz, 2H), 7.13 (d, J = 9.0 Hz, 1H), 8.28 (dd, J = 2.5, 9.0 Hz, 1H), 8.83 (d, J = 2.5 Hz, 1H), 9.36 (t, J = 5.0 Hz, 1H), 12.85 (bs, 2H); 13C-NMR (100.6 MHz, DMSO-d6): δ 41.6, 116.7, 118.6, 125.8, 129.1, 139.6, 164.5, 166.3, 171.0; HRESMS (m/z): [M – H]- calcd. for C9H7N2O6 239.0304; found 239.0302.

N-(5-amino-2-hydroxybenzoyl)glycine, 24.2,14 Brown solid: Mp: 302 °C decomp, lit. mp: 297 °C decomp, lit. mp: 184 ºC decomp. 1H-NMR (400 MHz, DMSO-d6): δ 3.94 (d, J = 5.5 Hz, 2H), 6.68-6.72 (m, 2H), 7.06-7.08 (m, 1H), 8.93 (t, J = 5.5 Hz, 1H), 11.02 (bs, 1H); 13C-NMR (100.6 MHz, DMSO-d6): δ 42.05, 113.9, 117.0, 118.3, 121.5, 141.4, 150.7, 168.9, 172.0; HRESMS (m/z): [M – H]- calcd. for C9H9N2O4 209.0562; found 209.0558.

Preparation of compounds 25 and 26 for the coupling with appropriate anhydride:
Commercially available 4-methoxyanisol was coupled with the appropriate anhydride using aluminium trichloride in dichloromethane, to provide hydroxyphenyl fumarate and hydroxyphenyl succinate 26 and 25 (Scheme 4).

\[
\text{OMe} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{OH} \quad \text{OH} \quad \text{OH} \quad \text{OH} \\
\quad \text{or} \\
\text{OH} \quad \text{O} \quad \text{OH} \quad \text{OH} \\
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Scheme 4. Reagents and condition: (i) AlCl₃, CH₂Cl₂, reflux, 1 h.

4-(2,5-Dihydroxyphenyl)-4-oxobutanoic acid, 25.15 Succinic anhydride (362 mg, 3.62mmol) and anhydrous aluminium trichloride (AlCl₃) (965 mg, 7.24 mmol) were dissolved in 1,2-dichloroethane (DCE) (10 ml) and heated at 50°C for 15 min. A solution of 1,4-dimethoxybenzene (500 mg, 3.62 mmol, 1eq) in 1,2-dichloroethane (DCE, 5 ml) was added dropwise and the reaction mixture was then heated up to reflux for 1h. The reaction was poured into a mixture of HCl (10N) and crushed ice, extracted with EtOAc (2 x 50 ml), washed with brine (2 x 50 ml), dried over anhydrous MgSO₄ and evaporated in vacuo. The crude residue was dissolved in DCE (5 ml) and was added to a suspension of AlCl₃ (965 mg, 7.24 mmol, 2eq) in DCE (10 ml) and refluxed for 1h. The reaction mixture was worked up as described above. The crude residue was purified by flash chromatography using CH₂Cl₂/MeOH/acetic acid from (98:2:1) to (95:5:1) ratio. The previous purification step was repeated twice to match purity requirement, affording 146 mg (19.2 %) of the desired product as a brownish solid. Mp 159-162 °C. 1H-NMR (400 MHz, DMSO, 1H-NMR variable temperature experiments indicated that conformational isomerism affected the observed integration values of the signals at 3.25-3.19 ppm and the overall appearance of the spectrum. Only one set of 1H-NMR signals is reported): δ 11.25 (br, 1H), 7.21 (d, J = 3.0 Hz, 1H), 6.99 (dd, J = 3.0, 9.0 Hz, 1H), 6.81 (d, J = 9.0 Hz, 1H), 3.25-3.19 (m, 1H), 2.58-2.55 (m, 1H); 13C-NMR (100.6 MHz, DMSO): δ 204.4,
174.6, 154.1, 150.3, 121.0, 119.2, 115.3, 35.1, 28.5; HRMSES (m/z): [M-H]^- calcd for C_{10}H_{9}O_{5}, 209.0450; found, 209.0449

(2E)-4-(2,5-Dihydroxyphenyl)-4-oxobut-2-enoic acid, \(26^{15}\). Maleic anhydride (0.71 g, 7.24 mmol) and anhydrous aluminium trichloride (AlCl_3) (1.93 g, 14.475 mmol) were dissolved in DCE (10 ml) and heated at 50°C for 15 min. A solution of 1,4-dimethoxybenzene (1.00 g, 7.24 mmol) in DCE (5 ml) was added dropwise and the reaction mixture was then heated up to reflux for 1h. The reaction was poured into a mixture of HCl (10N) and crushed ice, extracted with EtOAc (2 x 50 ml), washed with brine (2 x 50 ml), dried over anhydrous MgSO_4 and evaporated \textit{in vacuo}. The crude residue was dissolved in DCE (5 ml) and was added to a suspension of AlCl_3 (1.93 g, 14.475 mmol, 2eq) in DCE (10 ml) and refluxed overnight. The reaction mixture was worked up as described above. The crude residue was purified by silica gel chromatography using EtOAc/CH_2Cl_2/MeOH/Acetic acid from (0:90:10:1) to (10:80:10:1) ratio affording 308 mg (20.5 %) of the desired product as an orange solid. Mp: 183-187 °C. \(^{1}H\)-NMR (400 MHz, MeOD): \(\delta\) 7.98 (d, \(J = 15.5\) Hz, 1H), 7.26 (d, \(J = 3.0\) Hz, 1H), 7.08 (dd, \(J = 3.0, 9.0\) Hz, 1H), 6.86 (d, \(J = 15.5\) Hz, 1H), 6.86 (d, \(J = 9.0\) Hz, 1H); \(^{13}C\)-NMR (100.6 MHz, DMSO-\(d_6\), the \(^{13}C\) signal corresponding to one of the predicted C=O signals was not observed under these experimental conditions.): \(\delta\) 193.5, 156.6, 150.0, 136.1, 133.2, 126.0, 119.9, 118.9, 114.5; HRMSES (m/z): [M-H]^- calcd for C_{10}H_{7}O_{5}, 207.0293; found, 207.0286.

**Biological assays**

**Materials and methods:**
Preparations of purified recombinant forms of N-terminally truncated PHD2, amino acids 181-426 (tPHD2)\(^{16}\) and FIH\(^{17}\) were as described. GST-HIF-1\(\alpha\) 786-826, used as an FIH substrate, was prepared as reported\(^{17}\) and HIF-1\(\alpha\) 556-574 (CODD 19mer peptide), used as a tPHD2 substrate, was purchased from Peptide Protein Research Ltd. (Fareham, UK). Tricarboxylic acid intermediates and pyruvate were purchased from Sigma-Aldrich or Riedel-de-Häenor Fluka.

**Synthesis of peptides for VCB HTRF assays:**
19-residue peptides DLDLEMLAxYIPMDDDFQL corresponding to residues 556-574 of human HIF-1\(\alpha\), where x was a proline analog (proline, 4R-hydroxyproline) were prepared using a solid phase peptide synthesiser (CS Bio CS336) using Rink amide linker, PL-AMS resin (Polymer Laboratories) and standard Fmoc/DIC/HOBt strategy. Peptides were N-terminally biotinylated using biotin \(p\)-nitrophenyl ester (Novabiochem), final cleavage (CF\(_3\)COOH:phenol:H\(_2\)O:triisopropylsilane 88:5:5:2) gave peptides as C-terminal amides which were purified by preparative reversed-phase HPLC using a Vydac 218TP C18 10-
15u column (Grace Davison Discovery Sciences) and lyophilised. The mass of the peptide product was confirmed using a Micromass MALDI-TOF (Waters) mass spectrometer.

Electrospray ionisation-mass spectrometry of tPHD2.ligand complexes:
Electrospray ionisation-mass spectrometry (ESI-MS) of tPHD2.ligand complexes was performed using a Waters QToF\textsubscript{micro} quadrupole time-of-flight mass spectrometer equipped with an Advion NanoMate chip-based nano-ESI source. Protein (5 μM) and equimolar iron II and ligand samples were sprayed from aqueous ammonium acetate (25 mM, pH 7) using a NanoMate chip nozzle voltage of 1.55 kV. The QToF\textsubscript{micro} sample and extraction cone voltages were 80 and 2 V respectively (unless otherwise stated). Mass spectra were typically acquired over a range of m/z 500 to m/z 5000 for 30 to 60 s at a rate of 1 scan s\textsuperscript{-1} and deconvoluted using MaxEnt maximum entropy software. FIH assays were performed as described.\textsuperscript{18}

LC-ESI-MS/MS assays:
For the LC-mass spectrometric analyses, samples (8 μl) were injected onto a Jupiter C4 HPLC column (5μ, 150 x 0.5 mm, Phenomenex) using an Agilent 1100 capillary LC system and separated using 5 % acetonitrile/0.05 % formic acid as an aqueous mobile phase (flow rate of 15 μl/min). Mass analysis was performed from 0-35 min in the negative ion electrospray ionisation mode using a Waters QToF Micro mass spectrometer. Unless otherwise stated, the following conditions were used for all MS analyses: capillary voltage = 2384 V, cone voltage = 30 V, extraction cone voltage = 15 V, desolvation and nebulising nitrogen gas flow = 300 l/hour, desolvation temperature = 300°C, MS/MS collision voltage = 14 V.
Radiochemical $[^{14}C]$-labelled 2OG turnover assays:

2OG oxygenase activity was assayed in duplicate by measuring release of $[^{14}C]$-CO$_2$ from radiolabelled 2OG as described.$^{17}$ In summary, samples of the 2OG dependent oxygenase to be assayed were incubated with substrate in the presence of 80 μM Fe(II), 160 μM 2OG, 4 mM ascorbate, 1 mM DTT and 1 mM inhibitor in 50mM Tris-HCl, pH 7.5 at 37°C for 30 min. The final reaction volume was 100 μl. Reactions were stopped by the addition of 200 μl of methanol followed by a 30 min incubation on ice to allow collection of the $[^{14}C]$-CO$_2$. Where indicated samples were subjected to a one hour pre-incubation at room
temperature with either iron (+ buffer) or iron and ascorbate (+ buffer) and inhibitor where applicable before addition of missing cofactors followed by reaction at 37°C for 30 min. Anaerobic incubations were performed in a Belle Technology glove box under an argon atmosphere at < 0.5 ppm O₂.

Figure S3. Percentage of remaining activity of tPHD2 when incubated with 1 mM of the arylglycinate derivatives using a screening technique based on ^14CO₂ released without pre-incubation (Method A, blue) and subjected to 1 h pre-incubation with tPHD2 (Method B, green).

16, 23 and 24 exhibited inhibitory activity without pre-incubation, but with pre-incubation they apparently stimulated turnover. The apparent stimulation observed for some compounds may reflect the fact that 2OG and 2OG analogues are known to stabilise the PHD fold.¹⁹ We propose that tPHD2 undergoes partial denaturation (either by unfolding or aggregation) during the assays. Thus, 'weak' inhibitors (i.e. those that do not compete well with 2OG) (or compounds that do not inhibit) but which slow denaturation, may result in apparent stimulation of activity. With more potent inhibitors the inhibition activity out-competes any stabilisation effect.

Cell-based experiments procedure:

Human hepatocellular carcinoma cell line Hep3B, human osteosarcoma cell line U-2OS, and breast carcinoma cell line MCF7 were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) supplemented with 10 % fetal bovine serum (FCS) (Sigma), 2 mM L-glutamine (Sigma), and 50 IU/ml penicillin G/streptomycin (50 μg/ml) (Invitrogen), except for the human promonocytic cell line U-937, which was cultured in RPMI 1640 (Sigma), with identical supplements. Confluent cells were incubated
with medium containing the indicated inhibitor (dissolved in DMSO, stock concentration of 100 mM) or DMSO alone. To assay effects on TNF-α induced IKB-α degradation, cells were incubated with the indicated inhibitor for 45 min prior stimulation with TNF-α (10 ng/ml) (Sigma) for the final 15 min.

After incubation, cells were rinsed in ice-cold phosphate-buffered saline (PBS) and subsequently lysed in Nonidet P-40 buffer (20 mM HEPES pH 7.9, 420 mM NaCl, 0.4 % Nonidet P-40, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT), supplemented with cOmplete Protease Inhibitor Cocktail (Roche Applied Science). The protein concentrations were determined using a Bio-Rad protein assay (Bradford Reagent), and extracts were normalised to protein content. Whole cell lysate was resolved on a 7.5 or 10 % SDS-PAGE and then transferred to polyvinylidene difluoride membrane (Millipore). Primary antibodies used were mouse anti-HIF-2α (clone 190b), mouse anti-HIF-1α (BD Transduction Laboratories, clone 54), mouse anti-β-tubulin (Sigma), and mouse anti-CA9 antibody M7521 kindly provided by Silvia Pastorekova, the mouse anti-IκBα antibody was from R.T. Hay (University of Dundee, U.K.).
Figure S4. HIF-1α stabilisation monitored by immunoblotting staining SDS-PAGE gel. a) Effect of different concentrations of 3, 34 and 2 after 1 h, 6 h and overnight incubation with Hep3B cell lines on the HIF-1α level; b) Effect of high concentrations of 3, 34 and 2 after 6 h incubation with Hep3B cell lines on the HIF-1α level; c) Effect of different concentrations of 35 after 1 h, 2h, 6 h and o/n incubation with Hep3B cell lines on the HIF-1α level; d) Dose-effect experiment of 30 on Hep3B after 6 h incubation.

Urine extraction procedure and sample preparation:

Experiments were carried out by a modification of a previously reported method. About 10 h prior to the analysis, a dose of 1 g of pure soluble aspirin (ASPRO CLEAR®, 2 × tablets each containing 500 mg of aspirin) was ingested by one of us (B.M.L.) in full stomach. Urine (10 ml, taken from the first urine produced after overnight sleep) was neutralised to ca. pH 8.5 using a 0.2 M solution of NaOH, shaken for 5 min with a mixture of EtOAc/Et2O (14 mL/1 mL). 10 mL of the aqueous layer was separated, then
acidified by addition of concentrated HCl (1 mL). The acidified urine was then extracted with a mixture of EtOAc/Et₂O (14 mL/1 mL). The organic extract was then evaporated to dryness. The crude residue was then taken in DMSO (200 μM) and diluted to 2 mL with MilliQ purified water.

HPLC purification in preliminary analyses used a Waters 996 photodiode array detector, a Waters 600E system controller and a Waters 717 plus autosampler. Samples (50 μL) were injected onto a 5 μ Hypersil C18 HPLC column (250 × 4.60 mm, Phenomenex) and separated using 5 % acetonitrile + 0.05 % formic acid in H₂O as the mobile phase (Figure S5).

Figure S5.

LC-MS procedure:

For LC-MS analyses, the reaction mixture was injected onto a 5 μ Hypersil C18 HPLC column (250 × 4.60 mm, Phenomenex) using a Waters Alliance 2790 analytical HPLC system and separated using 5 % acetonitrile/0.05 % formic acid as a mobile phase over a period of 90 min at a flow rate of 1 ml/min. The flow from the column was analysed by a Waters LCT electrospray TOF mass spectrometer operated in the negative ion electrospray mode using nitrogen as a nebulising and desolvating gas (400 l/hr) with a sample cone voltage of 24 V.
Figure S6. LC-MS analyses (negative ion mode) conducted on (a) a urine sample before aspirin ingestion and (b) a urine sample after aspirin ingestion. Mass extraction chromatograms for: (c) 137 Da, (e) 178 Da, (g) 194 Da and (i) 210 Da from spectrum a; and (d) 137 Da, (f) 178 Da, (h) 194 Da and (j) 210 Da from spectrum b are shown.
Figure S7. HPLC-MS analyses monitoring at 210 Da for (a) a mixture of synthetic 14-17, (b) human urine sample without aspirin ingestion, (c) urine sample after aspirin ingestion, and (d) urine sample after aspirin ingestion doped with 0.05 μM of 14.

Bioinformatic analyses on the uPAR gene upregulated by aspirin

Aspirin has recently been shown to upregulate expression of the uPAR (urokinase type plasminogen activator receptor) gene; resulting in increased migration of HCT116 cells. Expression array data from the MCF-7 breast cancer cell line on upregulation of genes by hypoxia and with the oxygenase inhibitor dimethyloxalylglycine has also been reported. Interestingly, the uPAR gene (Hugo Gene Nomenclature Official Name: PLAUR) was strongly upregulated by both hypoxia and the 2OG oxygenase inhibitor (4.24-fold and 5.00-fold, respectively). In the uPAR gene 5’ promoter region, we located two hypoxia-response elements (HREs), both putative HIF transcription factor DNA binding sites, at -301 to -306 (AACGTG) and -130 to -135 (TGCGTG) from the start codon (ENSEMBL Transcript ID: ENST00000221264), flanking a c-Fos promoter (-236 to -229, ATGAGTCA), consistent with the reported downregulation of uPAR mRNA to a ratio of 0.60 by HIF-1α siRNA. Interestingly, both identified HREs are within the region corresponding to -1 to -398 of the uPAR promoter which has been identified to show maximum responsiveness to aspirin treatment and to be sufficient for the aspirin-induced up-regulation of uPAR. While we do not provide evidence that aspirin does indeed upregulate the uPAR gene via a HIF-related mechanism, our analyses highlight the possibility that this may be the case and suggest further study may be worthwhile.

Time-resolved fluorescence resonance energy transfer (TR-FRET) VCB assay:

To analyse binding of biotinylated peptides to ternary VCB (pVHL, Elongins C and B) complex, a modified homogeneous TR-FRET assay was used. Assays were performed in duplicate and detected
using three repeats per well on an EnVision Multilabel plate reader (PerkinElmer) at 25 °C. The data output ("HTRF signal") is the ratio of the 665 nm and 615 nm emission signals resulting from the 615 nm excitation of streptavidin-allophycocyanin and the 320 nm excitation of Eu³⁺, respectively, multiplied by 10,000. The percentage of control was determined by comparing the signal from hydroxylated peptide substrate in the enzyme reaction containing inhibitor compound with that from tPHD2 enzyme without inhibitor, and no enzyme.

Figure S8. tPHD2 inhibition assay using the TR-FRET VCB binding assay with compounds 10 and 14 (left and right panel, respectively); the determined IC₅₀ values were 113±1.23 and 27.41±1.33 μM, respectively. 95 % confidence intervals were 74.92-170.6 and 15.54-48.36 μM for 10 and 14, respectively.

References

1 KEGG PATHWAY Database-tryptophan metabolism.
http://www.genome.ad.jp/kegg/pathway/map/map00380.html


8 Compound commercially available from SinoChemexper Product List.


10 Compound commercially available from Acros Organic.

11 Compound commercially available from Ambinter Stock Screening Collection in milligram quantities.


