Supporting Information

Aspartate/asparagine-β-hydroxylase: a high-throughput mass spectrometric assay for discovery of small molecule inhibitors

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Supporting Figure S1. The synthetic AspH substrate cyclic peptide hFX-EGFD186-124-4Ser used in this work. The sequence of the hFX-EGFD186-124-4Ser peptide is derived from the reported human AspH substrate human coagulation factor X (hFX)\(^1\). Cystine sulfurs are in green; numbering is according to the sequence of hFX. (1) The canonical (Cys 1–3, 2–4, 5–6) EGFD1 disulfide pattern of hFX bearing the consensus sequence (orange/red) for AspH-catalysed Asp103\(_\text{hFX}\)-residue (red) hydroxylation. hFX EGFD1 with the canonical disulfide pattern is not a substrate for isolated AspH; (2) The non-canonical (Cys 1–2, 3–4, 5–6) EGFD1 disulfide pattern of hFX bearing the consensus sequence (orange/red) for AspH-catalysed Asp103\(_\text{hFX}\)-residue (red) hydroxylation. The non-canonical hFX EGFD1 is a substrate for isolated AspH\(^1\); (3) The hFX-EGFD186-124-4Ser peptide bearing the consensus sequence (orange/red) for AspH-catalysed Asp103\(_\text{hFX}\)-residue (red) hydroxylation. The hFX-EGFD186-124-4Ser peptide is a substrate for isolated AspH\(^1\). Substituted residues are in light blue.
Supporting Figure S2. Comparison of 2OG oxygenase protein substrate complex structures with pyridine-2,4-dicarboxylic acid (2,4-PDCA) bound. Colour code: grey: AspH; yellow: carbon-backbone of 2,4-PDCA; orange: carbon-backbone of the hFX-EGFD166-124-4Ser peptide (Supporting Figure S1); cyan: lysine-specific demethylase 4B (KDM4B); pink: carbon-backbone of the H3K9me3 peptide; violet: Mn; brown: Ni; red: oxygen; blue: nitrogen. w: water.

In the structures of AspH:2,4-PDCA (1; PDB ID: 5JTC) and KDM4B:2,4-PDCA (2; PDB ID: 4LXL) the binding of the 2,4-PDCA C-2 and C-4 carboxylates to protein residues differs significantly. In the case of AspH, the 2,4-PDCA C-2 carboxylate is positioned to interact with Arg688AspH and His690AspH (both 2.8 Å); the 2,4-PDCA C-4 carboxylate is positioned to form a salt bridge with Arg735AspH (2.4 and 3.1 Å) and is positioned to interact with Ser668AspH (2.7 Å). In the case of KDM4B, the 2,4-PDCA C-2 carboxylate is positioned to interact with Lys242KDM4B (3.0 Å), and faces towards the substrate binding pocket close to the substrate K9me3 group; the 2,4-PDCA C-4 carboxylate is positioned to interact with Tyr133KDM4B and Lys207KDM4B (2.6 and 2.7 Å). The spatial orientations of Tyr133KDM4B and Lys207KDM4B is apparently determined by interactions with Asn281KDM4B (3.1 and 2.9 Å). In addition to differences in their binding of 2,4-PDCA, the active sites of AspH and KDM4B differ with regard to both the number of residues interacting with the active site metal and the modes of substrate binding.
Supporting Figure S3. Comparison of selected human 2OG oxygenase crystal structures in complex with pyridine-2,4-dicarboxylic acid (2,4-PDCA). Colour code: grey: AspH; green: factor inhibiting hypoxia-inducible transcription factor (FIH); salmon: nucleolar protein 66 (NO66); cyan: Jmjc lysine-specific demethylase 4B (KDM4B); slate blue: fat mass- and obesity-associated protein (FTO); magenta: AlkB homolog 5 (Alkbh5); yellow: carbon-backbone of 2,4-PDCA; violet: Mn; orange: Fe; brown: Ni; metallic: Zn; red: oxygen; blue: nitrogen.

Comparison of the AspH:2,4-PDCA (1, PDB ID: 5JTC), FIH:2,4-PDCA (2, PDB ID: 2W0X)\(^2\), NO66:2,4-PDCA (3, PDB ID: 4DIQ), KDM4B:2,4-PDCA (4, PDB ID: 4LXL), FTO:2,4-PDCA (5, PDB ID: 4IE0)\(^4\), and Alkbh5:2,4-PDCA (6, PDB ID: 4NRQ)\(^4\) crystal structures reveals that the precise binding modes of 2OG oxygenases to 2,4-PDCA differ significantly. Between 1 and 3 residues interact directly with the either one or both of the two 2,4-PDCA C-4 carboxylate oxygen atoms; 1 to 2 protein residues interact directly with either one or both of the two 2,4-PDCA C-2 carboxylate oxygen atoms; metal ion coordination sites not occupied by protein residues can be occupied by water molecules (protein ligands and water molecules are not shown for clarity). Knowledge of the different binding modes of 2OG oxygenases with 2,4-PDCA (and related compounds) might be exploited for the design of derivatives that selectively inhibit specific sets of 2OG oxygenases.
Supporting Table S1. Summary of the 48 small-molecules of the library of pharmacologically active compounds (LOPAC, Sigma-Aldrich) which manifest >95% inhibition of AspH activity at a fixed inhibitor concentration (20 µM).

| AspH-Inhibitor                                      | Inhibition [%] |
|-----------------------------------------------------|----------------|
| Aurintricarboxylic acid                             | 103.8          |
| Suramin sodium salt                                 | 103.3          |
| 6-Hydroxy-DL-DOPA                                    | 103.1          |
| Iodoacetamide                                       | 102.0          |
| Guanidinyl-naltrindole di-fluorooacetate             | 101.7          |
| Morin                                               | 101.3          |
| 3,5-Dinitroctachol                                  | 101.2          |
| GW5074                                              | 101.1          |
| Calcimycin                                          | 100.8          |
| Tyrphostin 47                                       | 100.6          |
| SCH-202676 hydrobromide                             | 100.6          |
| Tyrphostin 51                                       | 100.6          |
| Clodronic acid                                      | 100.5          |
| Diethylenetriaminepentaacetic acid                  | 100.3          |
| Candesartan cilexetil                               | 100.1          |
| IPA-3                                               | 100.0          |
| Piceatannol                                         | 99.9           |
| MK-886                                              | 99.9           |
| Capsazepine                                         | 99.7           |
| Bisdemethoxycurcumin                                | 99.7           |
| Myricetin                                           | 99.4           |
| p-Benzoquinone                                      | 99.3           |
| 7,8-Dihydroxyflavone hydrate                        | 99.0           |
| Nordihydroguaiaretic acid from Larrea divaricata (creosote bush) | 98.9 |
|                                                     |                |
| AspH-Inhibitor                                      | Inhibition [%] |
| Tyroptisin AG 835                                   | 98.8           |
| (±)-6-Chloro-PB hydrobromide                        | 98.8           |
| Tyroptisin 23                                       | 98.7           |
| 3,4-Dihydroxyphenylacetic acid                      | 98.6           |
| Caffeic acid phenethyl ester                        | 98.5           |
| Daphnetin                                           | 98.5           |
| Hispidin                                            | 98.3           |
| Fenoldopam bromide                                  | 98.3           |
| Cephalosporin C zinc salt                           | 98.1           |
| Ro 41-0960                                          | 97.8           |
| Sildenafil (Viagra)                                  | 97.8           |
| Quercetin dihydrate                                 | 97.8           |
| 1,10-Phenanathroline monohydrde                     | 97.8           |
| (±)-Taxifolin                                       | 97.5           |
| Caffeic Acid                                        | 97.4           |
| PAC-1                                               | 96.1           |
| Apigenin                                            | 95.8           |
| (±)-Chloro-APB hydrobromide                         | 95.8           |
| Reactive Blue 2                                     | 95.8           |
| Tigecycline                                         | 95.3           |
| Benserazide hydrochloride                           | 95.2           |
| SKF 83959 hydrobromide                              | 95.1           |
| R(+)-6-Bromo-APB hydrobromide                       | 95.0           |
| NF 023                                              | 94.6           |
**Supporting Figure S4.** Differential scanning fluorimetry (DSF) assay. DSF assays were performed in independent duplicates as follows: The assay buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 50 μM NiCl₂) was gently mixed with SYPRO orange (1‰ v/v) and freshly thawed His₆-AspH₁₃₅₋₇₅₈ was added to a final AspH-concentration of 2 μM. The resulting orange solution was carefully mixed and pipetted into a 96-well Thermoscientific PCR-plate (19 μL per well). Finally, either an inhibitor solution (0.4 mM in DMSO) or a control sample (pure DMSO) was added to each well (1 μL per well, final inhibitor concentration of 20 μM) and the resulting solutions were gently mixed using a pipette. The plate was sealed with an optical tape (Bio-Rag, iCycler iQ), centrifuged (5 sec, 1000 rpm), then placed into a Stratagene Mx3005P PCR machine (Agilent Technologies). The PCR-machine was heated with a rate of 1° C per cycle (starting at an initial temperature of 25 °C; 70 cycles total). Data were analyzed using Microsoft Excel and GraphPad Prism following a literature protocol⁵.

Shifts (ΔTₘ) of the AspH melting temperature (Tₘ) are given with respect to DMSO controls. The validated AspH inhibitors 2,4-PDCA and NOG (entries 1 and 2), of which crystal structures in complex with AspH have been reported¹, show a notable increase in the AspH Tₘ, presumably by stabilizing the AspH active site. The presence of candesartan cilexetil (entry 3) in the assay buffer has no significant effect on the AspH Tₘ. PBIT (entry 4) seems to strongly destabilize AspH, potentially by binding to one or more of its nucleophilic residues. The positive effect of IOX1 (entry 5) on the AspH Tₘ is similar to that of NOG, suggesting that crystallization of AspH in the presence of IOX1 could afford co-crystals. Vadadustat seems to slightly destabilize AspH (entry 6).

| Entry | AspH-Inhibitor     | Δ[Tₘ-shift][°C] |
|-------|-------------------|----------------|
| 1     | 2,4-PDCA          | 3.5 ± 0.6      |
| 2     | NOG               | 2.8 ± 0.3      |
| 3     | Candesartan cilexetil | 0.2 ± 0.1     |
| 4     | PBIT              | -15.2 ± 1.4    |
| 5     | IOX1              | 2.8 ± 0.2      |
| 6     | Vadadustat        | -0.9 ± 0.2     |

a) Mean average of two independent runs (n = 2; mean ± standard deviation, SD).
**Supporting Figure S5.** Minimum significant ratio (MSR) analysis for the AspH inhibitors shown in Table 3. The mean ratio (MR), the MR confidence limits (RLs), the minimum significant ratio (MSR), and the limits of agreement (LsA) were calculated using Microsoft Excel\textsuperscript{6,7}. General reproducibility acceptance criteria for assay validation have been reported to be: MSR ≤ 3 and 0.33 < LsA < 3\textsuperscript{6}. The MR should ideally be 1 for a reproducible assay, the RLs should include 1\textsuperscript{6}.

Data of the small-molecule AspH inhibitors displayed in Table 3 (sample size: 22 compounds) have been analysed which is graphically depicted below. The values are in the range of those defined for an acceptable assay: MR = 0.96; RLs = 0.86 and 1.07; MSR = 1.57; LsA = 0.61 and 1.50.
**Supporting Table S2.** Crystallization conditions and data collection of the AspH:2,4-PDCA complex.

| PDB ID    | 5JTC         |
|-----------|--------------|
| **Crystallization** |               |
| Method    | Vapor diffusion, sitting drop (200 nL), protein-to-well ratio 1:1 |
| Temperature (K) | 277           |
| Crystallization conditions | 18 mg/mL His6-AspH315-758 (330 μM), 1 mM MnCl2, 2 mM pyridine-2,4-dicarboxylic acid, 200 mM NaBr, 20%wt/v PEG3350, 100 mM bis-tris propane, pH 8.5, 726 μM hFX-EGFD186-124-4Ser |
| AspH:substrate ratio | 1:2.2 (330:726 μM) |
| **Data collection** |               |
| Space group | P2₁2₁2₁       |
| Symmetry   | orthorhombic  |
| Cell dimensions: |               |
| a, b, c (Å) | 50.02, 91.66, 123.05 |
| α, β, γ (°) | 90.00, 90.00, 90.00 |
| X-Ray source | Synchrotron (Diamond Light Source I04) |
| Temperature (K) | 100           |
| Detector   | Pilatus 6M-F  |
| Resolution (Å) | 73.50-2.24 (2.30-2.24) |
| Rmerge     | 0.103 (0.895) |
| I / σI     | 19.8 (3.2)    |
| Completeness (%) | 100.0 (100.0) |
| Multiplicity | 13.1 (13.7)   |

a) Values in brackets indicate high resolution data shell.
Supporting Table S3. Refinement statistics for the AspH:2,4-PDCA complex.

| PDB ID | 5JTC |
|--------|------|

| Refinement   |       |
|--------------|-------|
| Resolution (Å) | 2.24  |
| No. reflections | 27971 |
| $R_{\text{work}} / R_{\text{free}}$ | 0.1963/ 0.2299 |

| No. atoms:            |       |
|-----------------------|-------|
| Protein               | 3580  |
| Ligand/ion            | 15    |
| Water                 | 177   |

| B-factors:            |       |
|-----------------------|-------|
| Protein               | 48.8  |
| Ligand/ion            | 43.7  |
| Water                 | 45.3  |

| R.m.s. deviations:    |       |
|-----------------------|-------|
| Bond lengths (Å)      | 0.003 |
| Bond angles (°)       | 0.749 |

Supporting References

1. Pfeffer, I. et al. Aspartate/asparagine-β-hydroxylase crystal structures reveal an unexpected epidermal growth factor-like domain substrate disulfide pattern. Nat. Commun. 10, 4910 (2019).

2. Conejo-Garcia, A. et al. Structural basis for binding of cyclic 2-oxoglutarate analogues to factor-inhibiting hypoxia-inducible factor. Bioorg. Med. Chem. Lett. 20, 6125-6128 (2010).

3. Aik, W. et al. Structural basis for inhibition of the fat mass and obesity associated protein (FTO). J. Med. Chem. 56, 3680-3688 (2013).

4. Feng, C. et al. Crystal structures of the human RNA demethylase Alkbh5 reveal basis for substrate recognition. J. Biol. Chem. 289, 11571-11583 (2014).

5. Niesen, F. H., Berglund, H. & Vedadi, M. The use of differential scanning fluorimetry to detect ligand interactions that promote protein stability. Nat. Protoc. 2, 2212-2221 (2007).

6. Eastwood, B. J. et al. The minimum significant ratio: a statistical parameter to characterize the reproducibility of potency estimates from concentration-response assays and estimation by replicate-experiment studies. J. Biomol. Screen. 11, 253-261 (2006).

7. Haas, J. V., Eastwood, B. J., Iversen, P. W., Devanarayan, V. & Weidner, J. R. Minimum significant ratio – a statistic to assess assay variabilily. In: Sittampalam GS, Grossman A, Brimacombe K, et al., editors. Assay guidance manual (2017). Bethesda (MD). (Available from: https://www.ncbi.nlm.nih.gov/books/NBK169432/)