HAO1 inhibitor development: Synthesis and screening of follow-up compounds from fragment hits by fluorescence-based activity assay - deposition 2 of 2

Introduction: The overarching aim of my project is to start development of small molecule inhibitors of HAO1 (hydroxy-acid oxidase 1/ glycolate oxidase) as a treatment for primary hyperoxaluria, a rare inborn error of metabolism in which the pathogenic driver is accumulation of glyoxylate, the product of HAO1. A more in depth introduction is provided on my Open Lab Notebook blog here: https://openlabnotebooks.org/project-overview-inhibition-of-hao1-to-treat-primary-hyperoxaluria-type-1/

Previous work: We performed a fragment screen of HAO1 by x-ray crystallography and confirmed binding of four fragment hits in three biologically relevant sites – active site, gating loop and oligomeric interface – by SPR. Fluorescence-based activity assay (Amplex Red) showed inhibition of HAO1 by two fragments that bound to the active site and one fragment that bound to the gating loop. These data are available as part of the SGC Target Enabling Package (TEP) found on Zenodo here: https://doi.org/10.5281/zenodo.1342618

Follow up compounds, selected using SAR-by-catalogue, have already been tested for three fragment hits from HAO1 screening campaign (two active site and one gating loop fragment) and the data for these are available on Zenodo here: https://doi.org/10.5281/zenodo.1477079

Aim of this work: To synthesise a small set of follow-up compounds, based on the oligomer interface fragment hit and test them by activity assay for inhibition of HAO1.

PART 1: SYNTHESIS OF FOLLOW UP COMPOUNDS

Selection of compounds: Based on fragment-bound structure, wanted to preserve chloro-pyrimidine and disconnect tetrahydrofuran, replacing it with bulkier synthons to try and disrupt HAO1 tetramer. The commercially available dichloro-pyrimidine and 15 diverse amines from our stock, greater than 150 Daltons in size, were selected for coupling through SNAr reation, following the scheme shown below.

Reaction conditions: 50 mg of dichloropyrimidine in 2 mL acetonitrile; added 2.2 molar equivlents of DIPEA; added 1.05 molar equivalents of amine building block. Reaction molarity: 0.168 M. Stirring at 660 rpm. Temperature and time differed between different amines (details in table below). Outcome assessed by analytical LC-MS – pH 6, 2mL/min, 3 min run time, ES+.

Purification: MeCN removed by evaporation (GeneVac, low bp method). Rtn 10 not purified further. Rtn 1 resuspended in DCM, transferred to silica and purified by flash chromatography (A/ Cyclohexane; B/ ethyl acetate; SNAP Ultra 10 g column; 0-100% B over 10 CV). Rtn 2 purification by flash chromatography unsuccessful (same conditions as for rtn 1) so purified by LC-MS. Remaining reactions resuspended in methanol (with added DMSO if needed to solubilise mixture) and purified by LC-MS – pH 6, 20 mL/min, 15 min run time, ES+, collection triggered by mass (product mass determined by analytical run, 2 uL injection from same vial). Fractions lyophilised using GeneVac (HPLC-Lyo method). Purity of selected fractions confirmed by analytical LC-MS and 1H-NMR.

Rtn no.	Amine structure	Rtn. °C/ h	Product structure	Product ID
1	HN, N, N	50/18	N, N, N, N, N, Cl	SM001-01
2	NH_2, N H, O	50/18	Cl, N, N, N, N, O	SM001-02
3	NH_2, N, OH, O	RT/18	N, O, O, N, N, N, Cl	SM001-03
4	NH, NH_2, N	50/18	N, Cl, N, N, N, N	SM001-04
5	O, HN, N, O	80/18	O, N, O, N, N, N, Cl	SM001-05
6	NH, NH_2, N	RT/18	N, N, N, N, N, Cl	SM001-06

7		RT/18		SM001-07
8		80/18		SM001-08
9		RT/18		SM001-09
10		RT/18		SM001-10
11		RT/18		SM001-11
12		160/18 (Micro-wave)		SM001-12
13		160/18 (Micro-wave)		SM001-13
14		160/18 (Micro-wave)		SM001-14
15		160/18 (Micro-wave		SM001-15

PART 2: ACTIVITY ASSAY

Working solutions:

Assay buffer: 50 mM sodium phosphate, pH 7.5; 200 mM potassium chloride, 2 mM magnesium chloride, 0.01% Triton-X.

Protein stock: 20 nM HAO1A-c002 (N-terminal 6-His tag followed by TEV protease cleavage site; M1-S368; diluted in assay buffer from 360 µM stock, purified by Ni-NTA IMAC and size exclusion chromatography and stored at – 80 °C)

Substrate stock: 72 µM glycolate in assay buffer

Amplex Red reagent: 100 µM Amplex Red dye (10 mM stock in 100% DMSO) and 0.2 U/mL horseradish peroxidase (10 U/mL stock in water) in assay buffer

Compounds: 40 mM stock solutions in 100 % DMSO

Methods:

Plate 1: compounds 1-5; Plate 2: compounds 6-10; Plate 3: compounds 12-14
Compounds 11 and 15 were excluded from analysis as they showed interference with the assay (oxidised Amplex Red without provision of hydrogen peroxide).

For each plate:
1. Prepare a 96-well PCR plate with a serial dilution of 5 compounds (12 concentrations; final of 1000, 750, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95 and 0.98 uM) with final volume of 1 uL; Additional row (12 wells) of 1 uL 100 % DMSO
2. Added 19 uL of 20 nM HAO1 to every well and incubated at RT for 30 min
3. Transferred to 384-well plate [Greiner-One flat bottom, small volume, HiBase, non-binding, 384-well black microplate] - 2.5 uL per well, transfer from each well of PCR-plate 6 times to fill 3 full rows of the 384-well plate
e.g. row A of 96-plate was added to A-C 1-24 of 384 well plate, giving triplicate wells for no substrate and activity measurement
4. Added 2.5 uL of buffer to all odd-numbered columns - these are the no substrate controls
5. After the 30 min incubation, added 2.5 uL of 72 uM glycolate to all even wells - these are for activity measurement, incubate at RT for additional 30 minutes
6. Added 5 uL of Amplex Red reagent to each well, covered with foil seal (to protect from light and air exposure), and incubated at RT for 20 min
7. On PherstarFS (FI 540 590 optic module): scanned entire plate to perform gain adjustment to 60%; read plate fluorescence
8. In excel:
    a. Calculated change in fluorescence for each set of replicates (activity measurement minus equivalent no substrate control)
    b. Calculate mean change in fluorescence for each triplicate
    c. Converted mean change for each inhibitor concentration to a % of the fluorescence change without an inhibitor (DMSO only control) - this is % activity
    d. Converted each measurement to % inhibition (100 - % activity)

e. Calculate standard deviation in fluorescence for each triplicate and convert to a % of the mean

9. In GraphPad Prism:

Enter data as: x = log (inhibitor concentration); y = MEAN (% inhibition), SD (% of mean)

Plot non-linear regression curve fit (log [Inhibitor] vs response, varaible slope, four parameters) to determine IC50s where possible

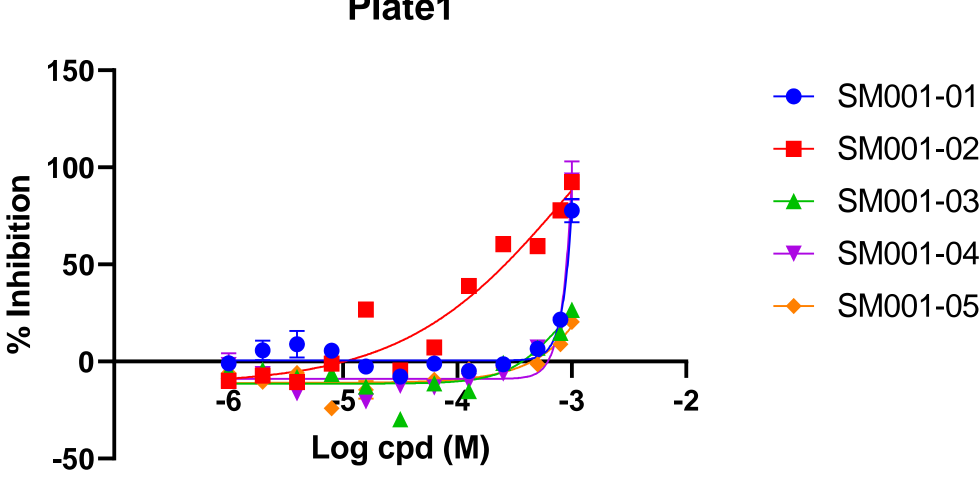
Results

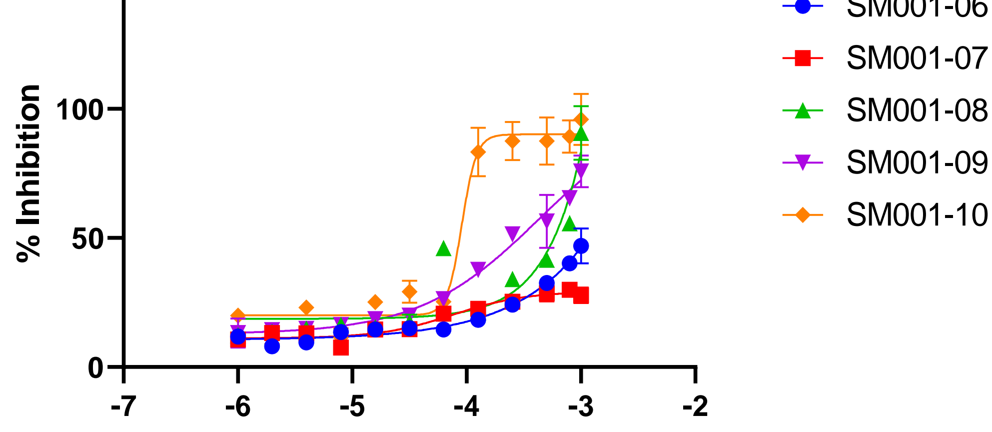
Inhibition data: Mean and standard deviations for each measurement, n = 3.

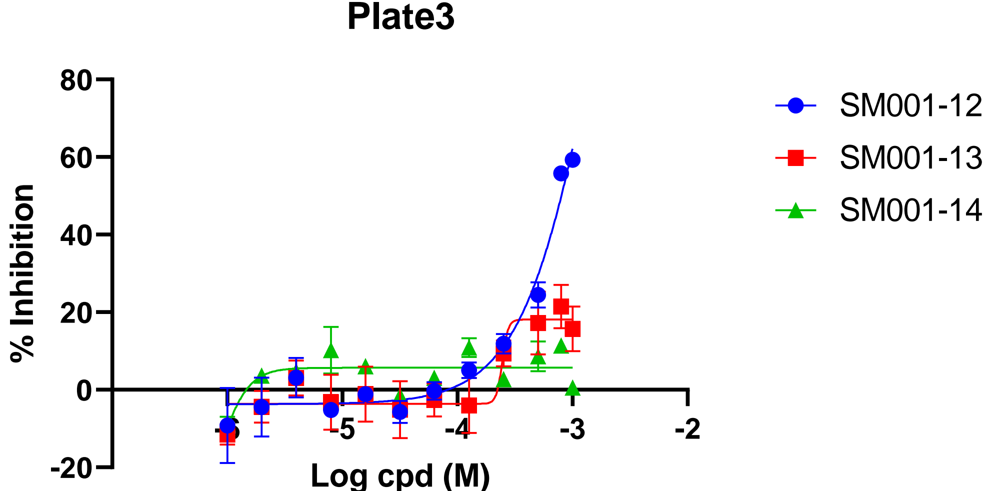
	SM001-01		SM001-02		SM001-03		SM001-04	
Log cpd (M)	% Inhibition	% SD	% Inhibition	% SD	% Inhibition	% SD	% Inhibition	% SD
-3.0	77.7	5.9	92.4	1.9	26.5	3.4	93.3	9.8
-3.1	21.5	0.7	77.9	1.7	14.7	3.6	18.9	2.8
-3.3	6.6	1.0	59.4	1.0	7.1	1.5	7.3	1.1
-3.6	-1.4	2.0	60.4	0.6	-0.8	1.1	-6.1	0.7
-3.9	-5.0	2.7	38.9	2.9	-15.2	1.5	-10.0	3.1
-4.2	-1.1	1.4	7.2	0.9	-11.3	1.7	-13.3	3.1
-4.5	-7.8	0.6	-4.5	0.9	-29.8	3.3	-12.4	0.3
-4.8	-2.6	0.7	26.8	0.1	-12.9	1.7	-20.4	1.0
-5.1	5.6	3.0	-1.0	1.7	-6.5	2.6	-3.6	1.1
-5.4	8.9	6.8	-10.5	0.8	-8.2	1.7	-16.3	2.2
-5.7	5.7	5.0	-7.3	0.4	-4.2	1.1	-6.4	1.9
-6.0	-0.9	0.4	-9.9	1.7	-4.7	0.5	-1.8	6.0
	SM001-05		SM001-06		SM001-07		SM001-08	
Log cpd (M)	% Inhibition	% SD	% Inhibition	% SD	% Inhibition	% SD	% Inhibition	% SD
-3.0	20.3	2.4	46.9	6.8	27.8	3.0	90.7	10.4
-3.1	8.9	1.3	40.2	2.3	30.0	1.1	55.7	0.5
-3.3	-1.5	2.6	32.5	2.9	28.5	3.1	41.6	1.9
-3.6	-3.3	1.4	24.1	1.8	25.3	1.6	34.1	1.7
-3.9	-3.9	1.6	18.3	2.6	22.6	1.4	21.2	2.8
-4.2	-9.5	2.3	14.5	3.0	20.7	1.0	46.1	2.2
-4.5	-8.9	3.4	15.1	2.3	14.7	1.8	17.3	3.0
-4.8	-14.6	4.5	14.5	2.2	14.6	2.5	15.3	2.3
-5.1	-24.1	3.8	13.6	2.0	7.6	0.0	16.3	1.7
-5.4	-5.9	1.9	9.5	2.1	13.1	2.8	14.8	1.6
-5.7	-10.5	2.8	8.1	2.7	13.2	2.3	13.5	2.2
-6.0	-5.9	2.8	11.9	2.4	10.7	1.4	12.3	1.1

	SM001-09		SM001-10		SM001-12		SM001-13	
Log cpd (M)	% Inhibition	% SD	% Inhibition	% SD	% Inhibition	% SD	% Inhibition	% SD
-3.0	75.8	6.2	95.9	9.8	59.3	0.9	15.7	5.7
-3.1	65.4	0.7	89.4	6.3	55.8	1.9	21.4	5.6
-3.3	56.5	10.3	87.5	9.1	24.4	3.3	17.2	8.1
-3.6	51.5	1.9	87.6	7.4	11.9	2.5	9.4	3.3
-3.9	37.7	0.7	83.4	9.4	5.1	2.0	-4.0	7.1
-4.2	26.3	2.2	25.3	1.2	-0.3	2.2	-2.6	4.3
-4.5	19.9	2.2	29.2	4.3	-5.7	2.9	-5.1	7.3
-4.8	18.6	0.8	25.2	1.5	-1.1	1.5	-1.1	7.1
-5.1	16.4	0.7	11.5	3.7	-5.2	1.1	-3.2	7.1
-5.4	14.8	1.0	23.1	0.9	3.1	5.1	3.1	4.6
-5.7	14.3	0.8	12.0	3.0	-4.5	7.6	-4.4	4.1
-6.0	13.2	5.7	20.0	0.6	-9.2	9.7	-11.5	2.6
	SM001-14							
Log cpd (M)	% Inhibition	% SD						
-3.0	0.5	1.8						
-3.1	11.4	1.5						
-3.3	8.6	3.8						
-3.6	2.7	1.6						
-3.9	10.8	2.5						
-4.2	3.0	1.5						
-4.5	-1.5	1.6						
-4.8	6.1	0.5						
-5.1	10.2	6.0						
-5.4	4.7	1.4						
-5.7	3.6	0.3						
-6.0	-9.8	2.8						

GraphPad curves:



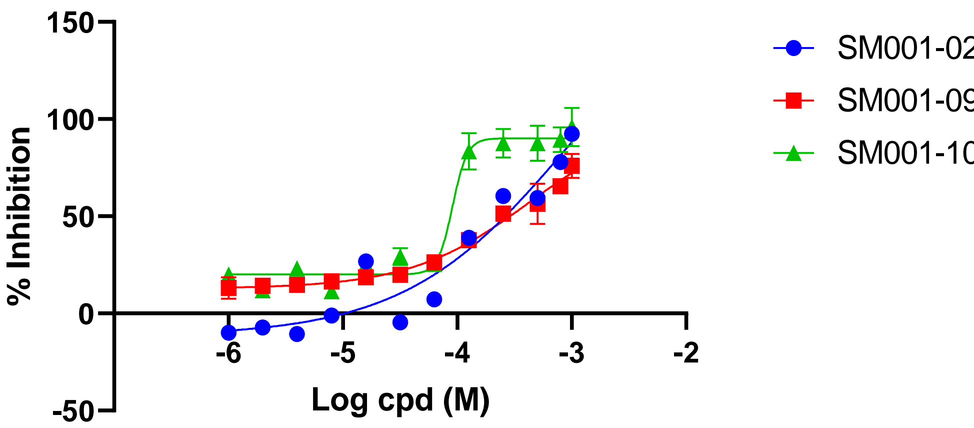




Analysis

Total of 3 follow-up compounds showed non-ambiguous curve fitting and high enough inhibition of HAO1 activity to allow IC50 determination. The appropriate fitted curves and calculated IC50 values are shown below.

Fitted curves:



IC50s: