HAO1 inhibitor development: Screening of follow-up compounds from fragment hits by fluorescence-based activity assay - deposition 1 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. This data is available as part of a SGC Target Enabling Package (TEP) on Zenodo here: <u>https://doi.org/10.5281/zenodo.1342618</u>

Aim of this work: To test follow up compounds, selected using SAR-by-catalogue, for three fragment hits from HAO1 screening campaign: two active site inhibitors and one gating loop inhibitor.

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 μ M 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

PART 1: SINGLE CONCENTRATION SCREEN

Aim: Screen all compounds at 1 mM to narrow down candidates for IC50 determination.

Methods:

- 1. Prepare a 96-well PCR plate with compounds, 1 uL at 40 mM in 100% DMSO; Additional row (12 wells) of 1 uL 100 % DMSO
- 2. Added 19 uL of 20 nM HAO1 to every well
- 3. Incubated at room temperature for 30 minutes
- 4. Transferred to 384-well plate [Greiner-One flat bottom, small volume, HiBase, non-binding, 384-well black microplate] 3 X 2.5 uL from each well
- 5. Added 2.5 uL of 72 uM glycolate to every well
- 6. Incubated at room temperature for 30 minutes
- 7. Added 5 uL of Amplex Red reagent to every well
- 8. Covered with foil seal (to protect from light and air exposure), and incubated at room temperature for 20 min
- 9. On PherstarFS (FI 540 590 optic module): scanned entire plate to perform gain adjustment to 60%; read plate fluorescence with excitation/emission of 570/585 nm
- 10. In excel:
 - a. Calculated mean fluorescence for each triplicate

- b. Converted mean to a % of the fluorescence without an inhibitor (DMSO only control) this is % activity
- c. Converted each measurement to % inhibition (100 % activity)
- d. Calculated standard deviation in fluorescence for each triplicate and convert to a % of the mean

<u>Results</u>

Control compounds:

Previous results quoted are from the HAO1 TEP document available here: https://doi.org/10.5281/zenodo.1342618

Compound ID	Structure	Previous result(s)	Current Inhibition at 1 mM (%)
Positive: CCPST	of the second se	IC50 190 uM	71.7 ± 1
Negative: INTERBIOSCREEN STOCK4S-65744		NT	0.6 ± 0.6
Fragment 1 (active site)	H ₂ N	85% inhibition at 10 mM	51.1 ± 0.9
Fragment 2 (active site)		37% inhibition at 10 mM	10.4 ± 1.6
Fragment 5 (gating loop)		19% inhibition at 10 mM	1.6 ± 0.3
Fragment 6 (interface)		<5% inhibition at 10 mM	0.3 ± 0.5

Active site follow-up compounds:

Structures: Arranged by plate layout i.e. row 1 is A, column 1 is 2



Assay results: All standard deviations were < 10% of the mean. Not reproduced here for clarity. Green fill compounds showed >50% inhibition, continued for IC50 determination. Yellow filled compounds were selected as controls in IC50 plate.

Plate well	Compound ID (Enamine)	Inhibition at 1 mM (%)	Plate well	Compound ID (Enamine)	Inhibition at 1 mM (%)	Plate well	Compound ID (Enamine)	Inhibition at 1 mM (%)
A2	Z55854425	44.5	D2	Z2757544766	4.0	G2	Z1536943320	64.1
A3	Z2235330312	2.1	D3	Z857666930	14.3	G3	Z1891775431	86.4
A4	Z248486708	67.2	D4	Z271099486	58.7	G4	Z2757544746	4.3
A5	Z2161617264	7.0	D5	Z1269137817	18.0	G5	Z3033976680	29.4
A6	Z1222424407	62.0	D6	Z2697514548	16.2	G6	Z2890506263	67.6
B2	Z55854313	56.3	E2	Z285197994	73.5	H2	Z1664348477	77.8
B3	Z1528412974	6.0	E3	Z57825360	17.6	H3	Z2895180259	0.7
B4	Z1889902438	28.8	E4	Z1173800291	46.0	H4	Z1916047016	29.2
B5	Z1724114279	5.3	E5	Z1813136853	21.4	H4	Z2335633115	16.7
B6	Z111423174	0.8	E6	Z1171742729	35.1	H6	Z90125187	71.6
C2	Z57282999	58.8	F2	Z1318255135	19.2			
С3	Z3063983225	9.6	F3	Z274568144	55.1			
C4	Z256709442	74.9	F4	Z1509171511	8.6			

C5	Z394875046	21.1	F5	Z55848625	37.0		
C6	Z1509533494	21.0	F6	Z1171742721	25.6		

Loop 4 follow-up compounds

Structures: Arranged by plate layout; compound ID is MolPort catalogue ID

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Plate_position A01 Compound_ID MolPort-001-808-009	Plate_position A03 Compound_ID MoIPort-005-508-245	Plate_position A05 Compound_ID MolPort-003-134-735	Plate_position A07 Compound_ID MolPort-004-213-546	Plate_position A09 Compound_ID MoIPort-005-541-261	Plate_position A11 Compound_ID MoIPort-005-589-535
001 tot	, àno			20 00	40 th
Plate_position A15 Compound_ID MolPort-005-729-746	Plate_position A41 Compound_ID MolPort-001-603-428	Plate_position A45 Compound_ID MolPort-000-262-661	Plate_position AC01 Compound_ID MolPort-003-280-787	Plate_position AC03 Compound_ID MoIPort-004-210-824	Plate_position AC05 Compound_ID MolPort-005-537-901
400 40	90P	2000		500	300
Plate_position AC07 Compound_ID MolPort-005-562-409	Plate_position AC11 Compound_ID MolPort-009-402-455	Plate_position AC17 Compound_ID MolPort-002-901-050	Plate_position AC37 Compound_ID MolPort-000-923-812	Plate_position AC47 Compound_ID MolPort-001-841-468	Plate_position C01 Compound_ID MolPort-001-502-185
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Plate_position E01 Compound_ID MolPort-003-334-620	Plate_position E03 Compound_ID MolPort-005-315-054	Plate_position E05 Compound_ID MolPort-004-122-552	Plate_position E07 Compound_ID MolPort-004-220-121	Plate_position E09 Compound_ID MolPort-001-573-341	Plate_position E11 Compound_ID MolPort-007-938-502
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Plate_position E15 Compound_ID MolPort-009-507-345	Plate_position E17 Compound_ID MolPort-006-845-110	Plate_position E29 Compound_ID MolPort-001-887-001	Plate_position E41 Compound_ID MolPort-002-097-342	Plate_position E45 Compound_ID MolPort-000-385-204	Plate_position G01 Compound_ID MolPort-001-541-506
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Plate_position I01 Compound_ID MolPort-001-684-354	Plate_position 105 Compound_ID MolPort-004-225-185	Compound_ID MolPort-005-533-351	Compound_ID MolPort-005-576-472	Plate_position I11 Compound_ID MolPort-005-628-799	Plate_position 115 Compound_ID MolPort-009-503-823
4000X		\$05 \$			
Plate_position I25 Compound_ID MolPort-002-231-869	Plate_position I37 Compound_ID MolPort-004-230-223	Plate_position I43 Compound_ID MolPort-002-009-211	Plate_position I45 Compound_ID MolPort-001-016-718	Plate_position K01 Compound_ID MolPort-001-543-988	Plate_position M03 Compound_ID MolPort-001-619-630
2000		0000		85 O.G.	40,X
Plate_position M07 Compound_ID MolPort-005-538-591	Plate_position M09 Compound_ID MolPort-005-581-473	Plate_position M11 Compound_ID MolPort-005-642-695	Plate_position M17 Compound_ID MolPort-001-538-468	Plate_position M19 Compound_ID MolPort-002-262-880	Plate_position M37 Compound_ID MolPort-004-232-834



Assay results: All standard deviations were < 10% of the mean. Not reproduced here for clarity. Green fill compounds showed >50% inhibition, continued for IC50 determination. Yellow fill compounds were selected as controls in IC50 plate.

Plate well	Inhibition at 1 mM (%)								
A01	6.1	AC37	18.0	101	5.7	M17	33.1	U07	88.8
A03	24.1	AC47	9.3	105	80.6	M19	57.4	U09	3.4
A05	9.5	C01	51.2	107	-3.6	M37	76.6	U25	17.9
A07	15.8	E01	72.0	109	48.4	M45	19.7	U37	19.3
A09	-5.1	E03	19.8	111	3.8	001	4.3	U47	2.9
A11	31.4	E05	0.8	115	18.2	Q03	3.5	Y01	13.0
A15	18.7	E07	9.9	125	101.8	Q07	14.9	Y03	21.6
A41	13.9	E09	23.3	137	61.8	Q09	58.6	Y05	63.9
A45	-1.3	E11	-2.5	143	13.9	Q11	3.2	Y07	35.3
AC01	20.3	E15	12.3	145	25.2	Q19	4.5	Y11	12.9
AC03	11.9	E17	-1.7	K01	21.6	Q37	2.6	Y17	17.6
AC05	16.5	E29	8.9	M03	7.7	Q45	42.4	Y21	45.6
AC07	18.5	E41	48.1	M07	12.3	S01	8.4	Y39	67.9
AC11	72.5	E45	1.7	M09	15.2	U01	24.7	Y45	57.0
AC17	16.2	G01	87.2	M11	8.4	U03	78.1	Y47	14.6

PART 2: IC50 DETERMINATION

Aim: Determine IC50 values for compounds that showed > 50% inhibition when screened at 1 mM.

Methods:

Plates 1-4: Loop 4 follow up compounds Plates 5-7: Active site follow up compounds

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

<u>Results</u>

Plate well:	A03		C01		E01		E03		E41	
Cpd	Mean %	SD								
logM	inhibition	(%)								
-3.0	92.6	2.2	31.8	1.6	94.2	6.8	1.3	0.7	65.9	1.7
-3.1	65.0	8.8	27.6	3.6	89.8	2.0	7.1	6.4	62.7	3.8
-3.3	44.6	1.2	24.7	9.3	85.3	4.7	5.4	5.8	58.7	3.3
-3.6	29.4	2.1	18.0	1.9	73.0	1.8	4.3	5.3	52.9	5.6
-3.9	26.5	0.8	14.7	2.6	53.6	1.8	-1.4	5.1	46.0	4.1

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

-4.2	12.0	1.6	9.2	2.2	31.2	1.4	-2.7	1.2	49.7	4.4
-4.5	8.2	4.1	5.0	10. 8	8.5	4.9	2.5	1.6	49.8	9.9
-4.8	13.5	10. 4	1.8	5.7	7.1	5.1	-0.1	1.5	44.4	1.1
-5.1	3.4	4.0	1.3	4.5	4.4	1.6	6.1	1.1	43.9	4.1
-5.4	3.2	1.6	-3.2	2.8	1.6	0.3	2.0	1.0	40.7	2.2
-5.7	-0.4	1.4	-0.1	4.3	2.5	1.5	-0.7	1.0	39.4	1.9
-6.0	-0.6	0.5	3.8	3.9	11.0	4.9	0.9	0.5	36.8	2.3
Plate	G01		105		125		137		M19	
well: Cod	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD
logM	inhibition	(%)	inhibition	(%)	inhibition	(%)	inhibition	(%)	inhibition	(%)
-3.0	97.0	8.8	94.8	1.6	97.8	2.0	-5.8	1.7	101.2	0.7
-3.1	92.0	1.2	85.0	3.6	95.4	4.7	8.0	6.4	79.9	3.8
-3.3	53.1	2.1	82.0	9.3	83.1	6.8	13.4	5.8	77.4	3.3
-3.6	45.6	0.8	63.3	1.9	84.9	1.8	11.8	5.3	72.7	5.6
-3.9	47.4	2.2	60.8	2.6	71.3	1.8	7.0	5.1	66.5	4.1
-4.2	33.4	1.6	60.3	2.2	56.7	1.4	3.4	1.2	34.7	4.4
-4.5	2.9	4.1	55.1	10. 8	36.2	4.9	-1.2	1.6	17.0	9.9
-4.8	0.1	10. 4	53.3	5.7	13.0	5.1	0.9	1.5	3.7	1.1
-5.1	0.8	4.0	15.7	4.5	6.9	1.6	1.8	1.1	-0.3	4.1
-5.4	1.3	1.6	19.1	2.8	5.5	0.3	2.2	1.0	4.5	2.2
-5.7	2.4	1.4	13.1	4.3	3.9	1.5	3.1	1.0	6.5	1.9
-6.0	3.9	0.5	9.8	3.9	2.4	4.9	0.5	0.5	4.9	2.3
Plata	N/27		009		1103		1107		1127	
well:	14137				005		007		037	
Cpd	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD
logM	inhibition	(%)	inhibition	(%)	inhibition	(%)	inhibition	(%)	inhibition	(%)
-3.0	72.1	2.9	88.8	6.2	16.5	2.5	99.4	7.4	66.5	1.5
-3.1	59.6	1.9	80.8	5.5	10.8	0.3	84.4	7.3	64.5	7.4
-3.3	50.6	0.9	70.6	8.4	5.1	1.9	86.8	8.8	29.0	0.8
-3.6	42.3	5.2	19.2	1.0	1.3	1.8	82.5	5.7	19.9	1.2
-3.9	40.5	3.7	5.7	5.2	-3.7	1.1	73.6	12.5	8.9	0.8
-4.2	30.2	1.4	6.3	1.6	-6.2	0.8	51.8	10.1	-4.6	0.5
-4.5	9.6	0.4	2.0	2.0	-8.3	1.7	7.7	6.5	-6.3	1.9
-4.8	5.6	2.5	2.5	2.7	-4.3	2.0	5.8	7.4	0.3	1.3

-5.1	0.2	1.9	2.4	2.1	-8.3	3.3	2.2	1.0	-5.1	0.3
-5.4	2.6	2.2	1.9	2.3	-6.1	2.5	-3.7	1.0	-13.4	1.6
-5.7	2.2	2.5	-0.2	1.6	-9.9	2.3	-4.9	1.0	-8.3	1.1
-6.0	0.0	2.0	-0.4	9.2	-8.7	2.2	-6.9	0.8	-13.4	0.7
Plate	Y05		Y07	•	Y39		Y45		AC11	
well:		60		60		<u> </u>	N/2222 0/	60	N4	60
Cpd logM	inhibition	SD (%)	inhibition	SD (%)	inhibition	SD (%)	inhibition	SD (%)	inhibition	SD (%)
-3.0	79.2	8.7	96.9	10. 1	62.3	0.8	74.4	1.9	93.8	8.1
-3.1	71.7	6.4	95.2	10. 2	63.0	3.4	50.5	5.2	70.8	9.3
-3.3	68.8	4.2	87.8	10. 5	59.0	7.4	37.2	8.7	63.9	7.5
-3.6	59.9	5.5	71.0	3.5	57.5	6.1	31.5	5.8	49.2	5.2
-3.9	60.0	7.2	51.3	9.1	50.8	3.4	18.9	3.7	40.0	5.2
-4.2	50.5	0.9	48.6	2.4	49.4	2.4	9.9	7.3	42.7	3.9
-4.5	31.8	8.1	22.0	6.9	49.6	1.7	9.4	2.2	32.1	3.3
-4.8	14.9	2.9	14.9	3.7	53.0	2.4	10.5	3.5	27.5	8.7
-5.1	12.9	3.7	9.3	6.0	52.3	1.7	3.5	3.0	28.1	10.2
-5.4	8.2	4.5	10.6	4.2	50.3	1.5	3.4	2.0	26.9	4.7
-5.7	6.4	4.5	11.2	5.3	35.6	2.8	1.2	1.7	20.2	7.2
-6.0	4.6	6.1	9.8	7.1	24.9	5.3	1.6	1.8	22.2	5.3
Plate well:	A4		A6		B2		B4		C2	
Cpd	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD
	inhibition	(%) 8 7	inhibition	(%)	inhibition	(%)	inhibition	(%)	inhibition	(%)
-3.1	70.8	6.4	95.4	3.1	57.7	7.4	48.9	5.2	69.9	9.3
-3.3	67.7	4.2	87.9	4.4	61.4	1.9	35.1	8.7	63.2	6.8
-3.6	58.6	5.5	70.1	3.5	56.1	6.1	29.3	5.8	52.1	3.0
-3.9	48.9	0.9	49.8	9.1	49.2	3.4	16.2	3.7	40.3	3.8
-4.2	45.3	6.8	43.3	9.6	47.8	2.4	7.6	3.5	37.2	3.2
-4.5	29.6	8.1	19.5	6.9	47.9	1.7	7.0	7.3	29.9	3.3
-4.8	12.2	2.9	12.1	3.7	51.5	2.4	6.5	2.2	24.5	4.7
-5.1	3.4	4.5	6.4	6.0	50.8	1.7	0.4	3.0	25.7	10.2
-5.4	1.5	6.1	7.7	4.2	48.7	1.5	0.2	2.0	25.1	8.7
-5.7			0.0	52	22 5	20	-1.6	1.8	10 7	53
L	10.0	3.7	8.3	5.5	55.5	2.0	-1.0	1.0	19.7	5.5
-6.0	10.0 5.2	3.7 4.5	6.9	7.1	22.4	5.3	-2.0	1.7	17.6	7.2

Plate	C4		D4		E2		E5		F3	
well:		1				1		1		
Cpd	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD	Mean %	SD
IOgivi	Inhibition	(%)	Innibition	(%)	innibition	(%)	Inhibition	(%)	Inhibition	(%)
-3.0	65.6	6.5	26.0	1.0	24.4	8.6	89.8	5.4	23.9	4.6
-3.1	64.4	3.4	21.6	3.5	17.8	2.3	87.2	3.6	5.9	0.9
-3.3	56.1	9.7	19.4	4.2	7.6	3.4	79.3	9.6	-3.1	0.4
-3.6	56.2	8.5	18.9	4.6	-1.4	2.0	74.5	2.4	-5.1	4.9
-3.9	52.6	2.5	11.5	4.2	-11.1	0.2	60.6	0.5	-5.1	4.2
-4.2	52.5	9.7	11.2	1.0	-58.6	2.5	40.8	6.2	-0.2	1.8
-4.5	45.8	1.5	9.1	1.9	-19.4	2.8	17.1	4.4	-8.6	3.3
-4.8	41.1	0.5	0.4	3.6	-20.6	0.8	-1.2	2.5	-5.6	3.8
-5.1	37.4	2.2	0.9	2.1	-13.4	3.4	-13.9	2.7	-7.9	1.9
-5.4	26.1	2.4	-6.3	2.3	-9.2	2.3	-10.8	0.7	3.8	3.2
-5.7	20.3	1.5	-7.3	4.5	-2.7	1.6	-7.7	1.1	3.0	2.7
-6.0	15.3	2.0	-5.0	4.0	3.1	2.3	-4.8	0.7	9.3	2.6
Plate well:	G2		G3		G6		H2		H6	
Plate well: Cpd	G2 Mean %	SD	G3 Mean %	SD	G6 Mean %	SD	H2 Mean %	SD	H6 Mean %	SD
Plate well: Cpd logM	G2 Mean % inhibition	SD (%)	G3 Mean % inhibition	SD (%)	G6 Mean % inhibition	SD (%)	H2 Mean % inhibition	SD (%)	H6 Mean % inhibition	SD (%)
Plate well: Cpd logM -3.0	G2 Mean % inhibition 88.8	SD (%) 5.0	G3 Mean % inhibition 91.9	SD (%) 1.5	G6 Mean % inhibition 90.5	SD (%) 3.2	H2 Mean % inhibition 82.3	SD (%) 3.4	H6 Mean % inhibition 92.3	SD (%) 6.3
Plate well: Cpd logM -3.0 -3.1	G2 Mean % inhibition 88.8 80.8	SD (%) 5.0 2.9	G3 Mean % inhibition 91.9 84.8	SD (%) 1.5 4.6	G6 Mean % inhibition 90.5 81.3	SD (%) 3.2 7.8	H2 Mean % inhibition 82.3 70.6	SD (%) 3.4 0.9	H6 Mean % inhibition 92.3 84.5	SD (%) 6.3 3.4
Plate well: Cpd logM -3.0 -3.1 -3.3	G2 Mean % inhibition 88.8 80.8 77.9	SD (%) 5.0 2.9 1.4	G3 Mean % inhibition 91.9 84.8 82.5	SD (%) 1.5 4.6 2.7	G6 Mean % inhibition 90.5 81.3 71.2	SD (%) 3.2 7.8 2.2	H2 Mean % inhibition 82.3 70.6 63.1	SD (%) 3.4 0.9 3.5	H6 Mean % inhibition 92.3 84.5 77.5	SD (%) 6.3 3.4 2.3
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6	G2 Mean % inhibition 88.8 80.8 77.9 63.6	SD (%) 5.0 2.9 1.4 1.4	G3 Mean % inhibition 91.9 84.8 82.5 70.9	SD (%) 1.5 4.6 2.7 2.5	G6 Mean % inhibition 90.5 81.3 71.2 62.5	SD (%) 3.2 7.8 2.2 0.4	H2 Mean % inhibition 82.3 70.6 63.1 55.1	SD (%) 3.4 0.9 3.5 3.0	H6 Mean % inhibition 92.3 84.5 77.5 67.6	SD (%) 6.3 3.4 2.3 2.5
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6 -3.9	G2 Mean % inhibition 88.8 80.8 77.9 63.6 47.9	SD (%) 5.0 2.9 1.4 1.4 4.2	G3 Mean % inhibition 91.9 84.8 82.5 70.9 63.5	SD (%) 1.5 4.6 2.7 2.5 1.4	G6 Mean % inhibition 90.5 81.3 71.2 62.5 56.2	SD (%) 3.2 7.8 2.2 0.4 0.9	H2 Mean % inhibition 82.3 70.6 63.1 55.1 49.1	SD (%) 3.4 0.9 3.5 3.0 0.8	H6 Mean % inhibition 92.3 84.5 77.5 67.6 64.3	SD (%) 6.3 3.4 2.3 2.5 1.6
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6 -3.9 -4.2	G2 Mean % inhibition 88.8 80.8 77.9 63.6 47.9 24.6	SD (%) 5.0 2.9 1.4 1.4 4.2 7.5	G3 Mean % inhibition 91.9 84.8 82.5 70.9 63.5 58.2	SD (%) 1.5 4.6 2.7 2.5 1.4 1.2	G6 Mean % inhibition 90.5 81.3 71.2 62.5 56.2 52.0	SD (%) 3.2 7.8 2.2 0.4 0.9 2.9	H2 Mean % inhibition 82.3 70.6 63.1 55.1 49.1 40.7	SD (%) 3.4 0.9 3.5 3.0 0.8 0.5	H6 Mean % inhibition 92.3 84.5 77.5 67.6 64.3 66.1	SD (%) 6.3 3.4 2.3 2.5 1.6 9.0
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6 -3.9 -4.2 -4.5	G2 Mean % inhibition 88.8 80.8 77.9 63.6 47.9 24.6 19.0	SD (%) 5.0 2.9 1.4 1.4 4.2 7.5 1.6	G3 Mean % inhibition 91.9 84.8 82.5 70.9 63.5 58.2 53.8	SD (%) 1.5 4.6 2.7 2.5 1.4 1.2 0.8	G6 Mean % inhibition 90.5 81.3 71.2 62.5 56.2 52.0 49.8	SD (%) 3.2 7.8 2.2 0.4 0.9 2.9 1.0	H2 Mean % inhibition 82.3 70.6 63.1 55.1 49.1 40.7 31.5	SD (%) 3.4 0.9 3.5 3.0 0.8 0.5 1.7	H6 Mean % inhibition 92.3 84.5 77.5 67.6 64.3 66.1 58.3	SD (%) 6.3 3.4 2.3 2.5 1.6 9.0 5.1
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6 -3.9 -4.2 -4.5 -4.8	G2 Mean % inhibition 88.8 80.8 77.9 63.6 47.9 24.6 19.0 13.3	SD (%) 5.0 2.9 1.4 1.4 4.2 7.5 1.6 1.5	G3 Mean % inhibition 91.9 84.8 82.5 70.9 63.5 58.2 53.8 49.8	SD (%) 1.5 4.6 2.7 2.5 1.4 1.2 0.8 3.0	G6 Mean % inhibition 90.5 81.3 71.2 62.5 56.2 52.0 49.8 49.5	SD (%) 3.2 7.8 2.2 0.4 0.9 2.9 1.0 3.9	H2 Mean % inhibition 82.3 70.6 63.1 55.1 49.1 40.7 31.5 17.7	SD (%) 3.4 0.9 3.5 3.0 0.8 0.5 1.7 0.8	H6 Mean % inhibition 92.3 84.5 77.5 67.6 64.3 66.1 58.3 56.5	SD (%) 6.3 3.4 2.3 2.5 1.6 9.0 5.1 3.3
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6 -3.9 -4.2 -4.5 -4.8 -5.1	G2 Mean % inhibition 88.8 80.8 77.9 63.6 47.9 24.6 19.0 13.3 11.7	SD (%) 5.0 2.9 1.4 1.4 4.2 7.5 1.6 1.5 1.7	G3 Mean % inhibition 91.9 84.8 82.5 70.9 63.5 58.2 53.8 49.8 27.4	SD (%) 1.5 4.6 2.7 2.5 1.4 1.2 0.8 3.0 3.1	G6 Mean % inhibition 90.5 81.3 71.2 62.5 56.2 52.0 49.8 49.5 49.8	SD (%) 3.2 7.8 2.2 0.4 0.9 2.9 1.0 3.9 3.9	H2 Mean % inhibition 82.3 70.6 63.1 55.1 49.1 40.7 31.5 17.7 15.3	SD (%) 3.4 0.9 3.5 3.0 0.8 0.5 1.7 0.8 1.1	H6 Mean % inhibition 92.3 84.5 77.5 67.6 64.3 66.1 58.3 56.5 48.7	SD (%) 6.3 3.4 2.3 2.5 1.6 9.0 5.1 3.3 6.5
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6 -3.9 -4.2 -4.5 -4.8 -5.1 -5.4	G2 Mean % inhibition 88.8 80.8 77.9 63.6 47.9 24.6 19.0 13.3 11.7 10.6	SD (%) 5.0 2.9 1.4 1.4 4.2 7.5 1.6 1.5 1.7 2.4	G3 Mean % inhibition 91.9 84.8 82.5 70.9 63.5 58.2 53.8 49.8 27.4 18.0	SD (%) 1.5 4.6 2.7 2.5 1.4 1.2 0.8 3.0 3.1 0.9	G6 Mean % inhibition 90.5 81.3 71.2 62.5 56.2 52.0 49.8 49.5 49.8 49.5	SD (%) 3.2 7.8 2.2 0.4 0.9 2.9 1.0 3.9 3.9 2.6	H2 Mean % inhibition 82.3 70.6 63.1 55.1 49.1 40.7 31.5 17.7 15.3 15.5	SD (%) 3.4 0.9 3.5 3.0 0.8 0.5 1.7 0.8 1.1 1.2	H6 Mean % inhibition 92.3 84.5 77.5 67.6 64.3 66.1 58.3 56.5 48.7 50.8	SD (%) 6.3 3.4 2.3 2.5 1.6 9.0 5.1 3.3 6.5 5.3
Plate well: Cpd logM -3.0 -3.1 -3.3 -3.6 -3.9 -4.2 -4.5 -4.8 -5.1 -5.4 -5.7	G2 Mean % inhibition 88.8 80.8 77.9 63.6 47.9 24.6 19.0 13.3 11.7 10.6 10.7	SD (%) 5.0 2.9 1.4 1.4 4.2 7.5 1.6 1.5 1.7 2.4 4.0	G3 Mean % inhibition 91.9 84.8 82.5 70.9 63.5 58.2 53.8 49.8 27.4 18.0 15.5	SD (%) 1.5 4.6 2.7 2.5 1.4 1.2 0.8 3.0 3.1 0.9 2.0	G6 Mean % inhibition 90.5 81.3 71.2 62.5 56.2 52.0 49.8 49.5 49.8 49.5 49.8 43.2 36.8	SD (%) 3.2 7.8 2.2 0.4 0.9 2.9 1.0 3.9 3.9 2.6 1.9	H2 Mean % inhibition 82.3 70.6 63.1 55.1 49.1 40.7 31.5 17.7 15.3 15.5 9.0	SD (%) 3.4 0.9 3.5 3.0 0.8 0.5 1.7 0.8 1.1 1.2 2.3	H6 Mean % inhibition 92.3 84.5 77.5 67.6 64.3 66.1 58.3 56.5 48.7 50.8 43.2	SD (%) 6.3 3.4 2.3 2.5 1.6 9.0 5.1 3.3 6.5 5.3 3.6

PLATE 1





Plate 3



Plate 5



<u>Analysis</u>

Total of 5 active site follow-up compounds and 8 gating loop follow-up compounds showed nonambiguous curve fitting and high enough inhibition of HAO1 activity to allow IC50 determination. The appropriate fitted curves and calculated IC50 values are shown below.

Well-fitted curves for active site FUPs:





Well-fitted curves for loop FUPs:



