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: https://doi.org/10.5281/zenodo.1342618

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 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

PART 1: SINGLE CONCENTRATION SCREEN

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

Methods:

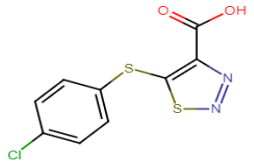
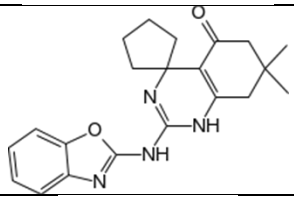
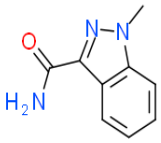
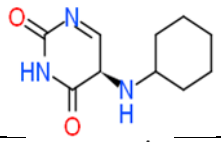
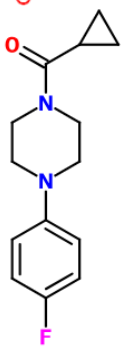
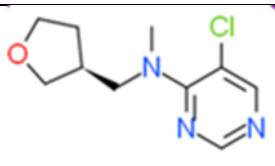
1. Prepare a 96-well PCR plate with compounds, 1 uL at 40 mM in 100% DMSO; Additional row (12 wells) of 1 uL 100 % DMSO
2. Added 19 uL of 20 nM HAO1 to every well
3. Incubated at room temperature for 30 minutes
4. Transferred to 384-well plate [Greiner-One flat bottom, small volume, HiBase, non-binding, 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

Results

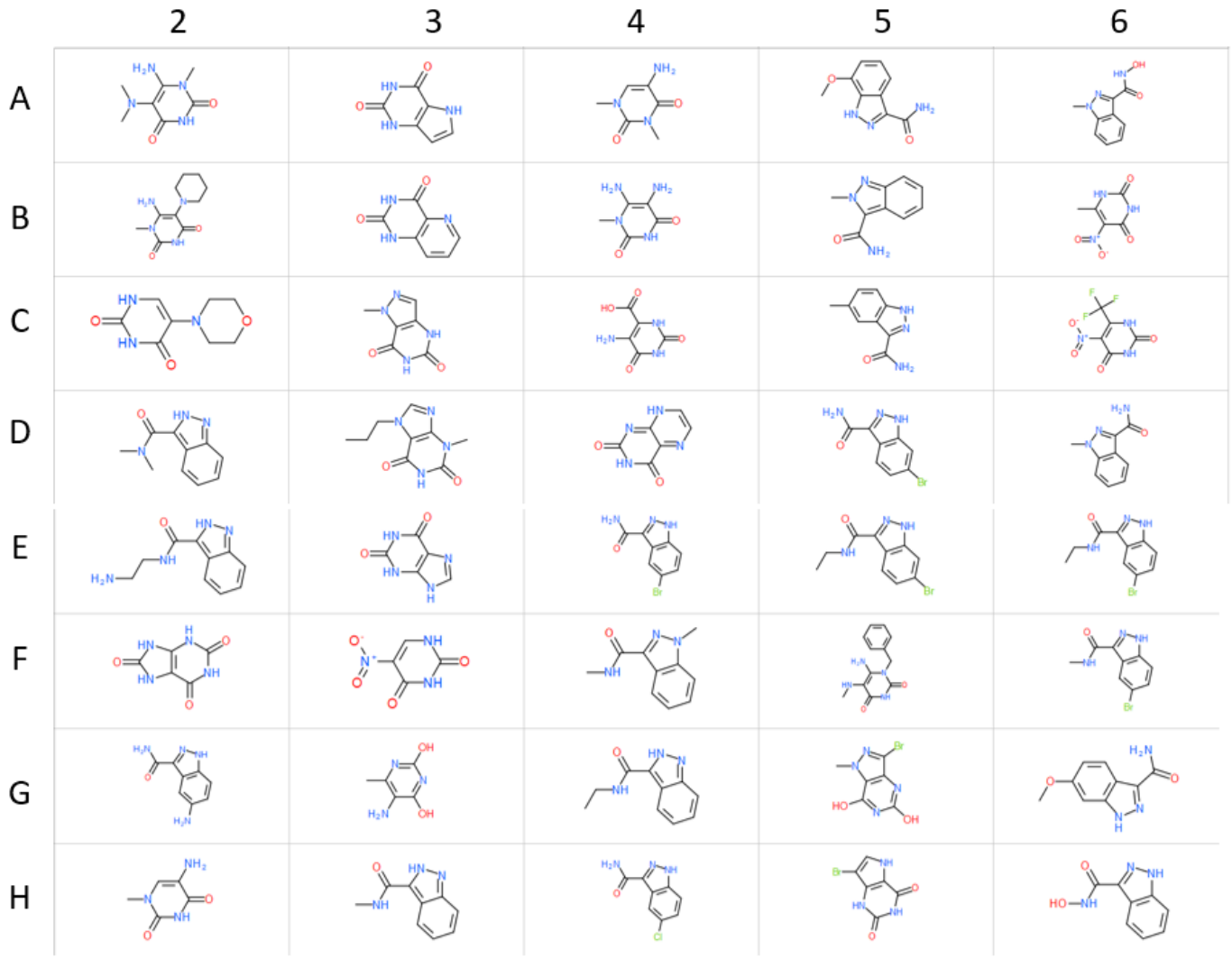
Control compounds:

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		IC50 190 uM	71.7 ± 1
Negative: INTERBIOSCREEN STOCK4S-65744		NT	0.6 ± 0.6
Fragment 1 (active site)		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



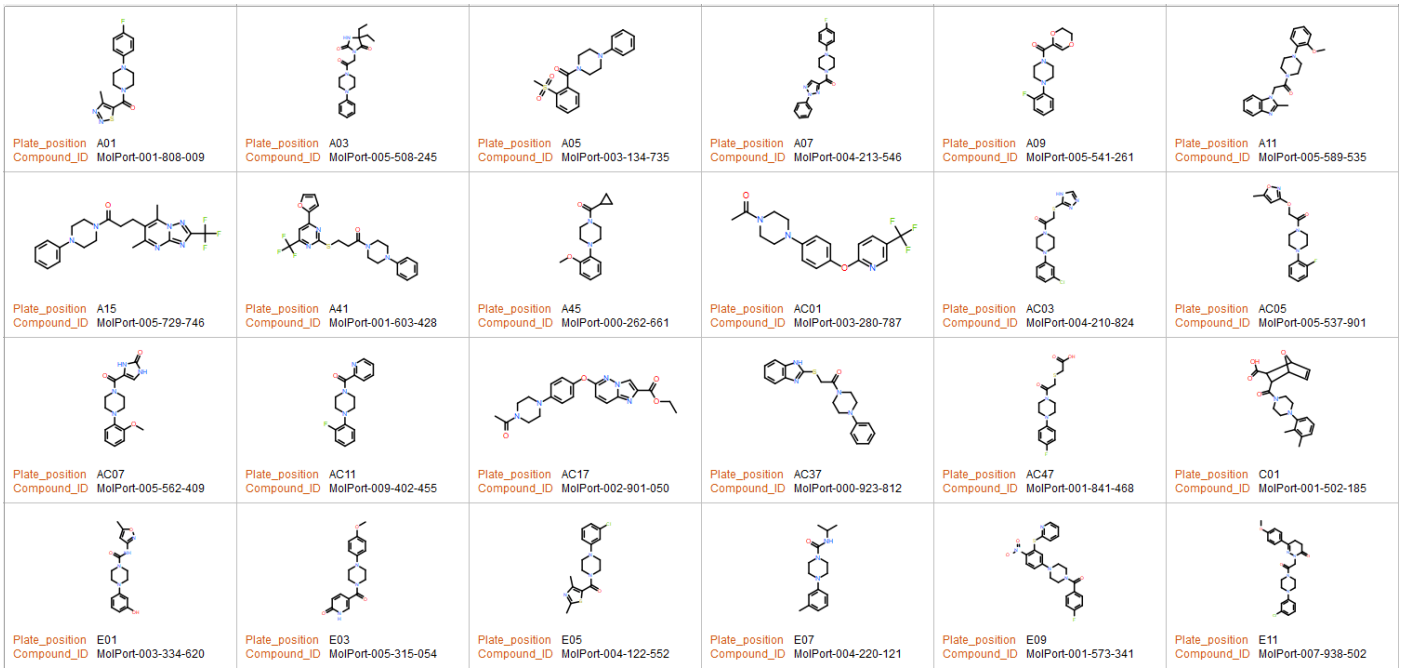
Assay results: All standard deviations were < 10% of the mean. Not reproduced here for clarity. Green fill compounds showed >50% inhibition, continued for IC50 determination. Yellow filled compounds were selected as controls in IC50 plate.

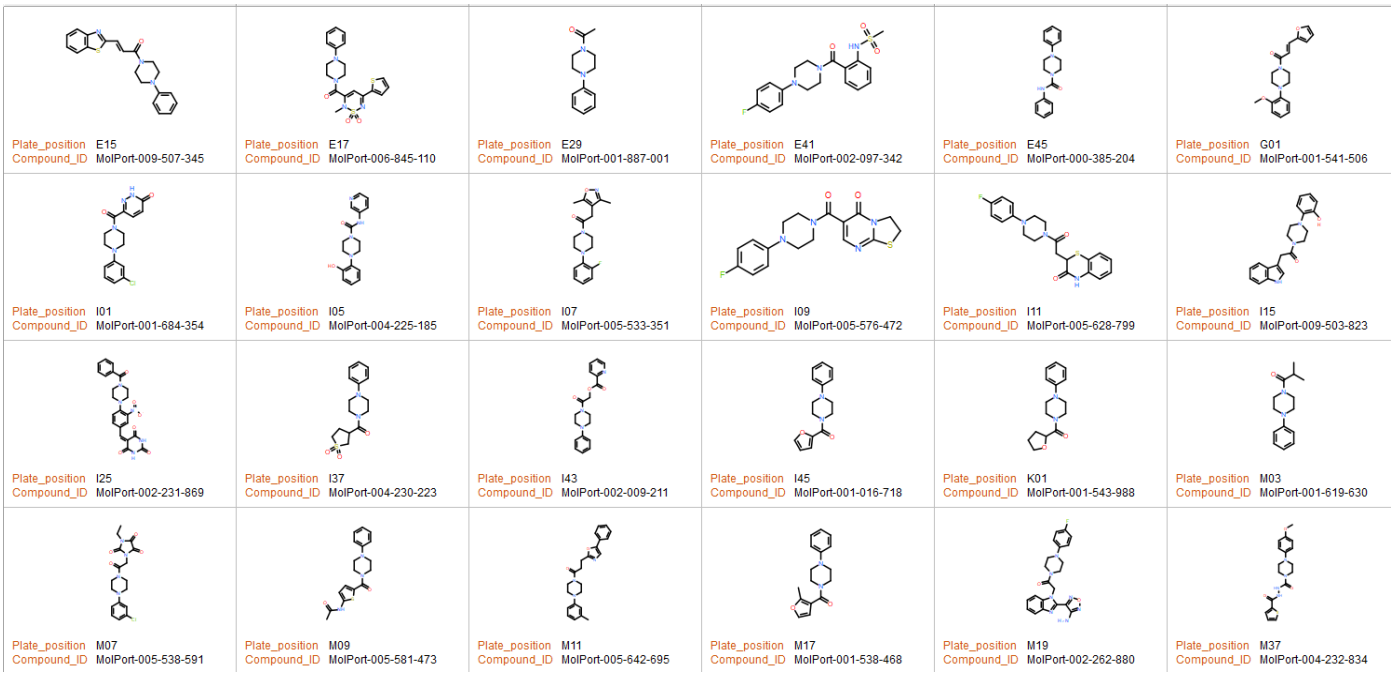
Plate well	Compound ID (Enamine)	Inhibition at 1 mM (%)	Plate well	Compound ID (Enamine)	Inhibition at 1 mM (%)	Plate well	Compound ID (Enamine)	Inhibition at 1 mM (%)
A2	Z55854425	44.5	**D2**	Z2757544766	4.0	**G2**	Z1536943320	64.1
A3	Z2235330312	2.1	**D3**	Z857666930	14.3	**G3**	Z1891775431	86.4
A4	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			
C3	Z3063983225	9.6	**F3**	Z274568144	55.1			
C4	Z256709442	74.9	**F4**	Z1509171511	8.6			

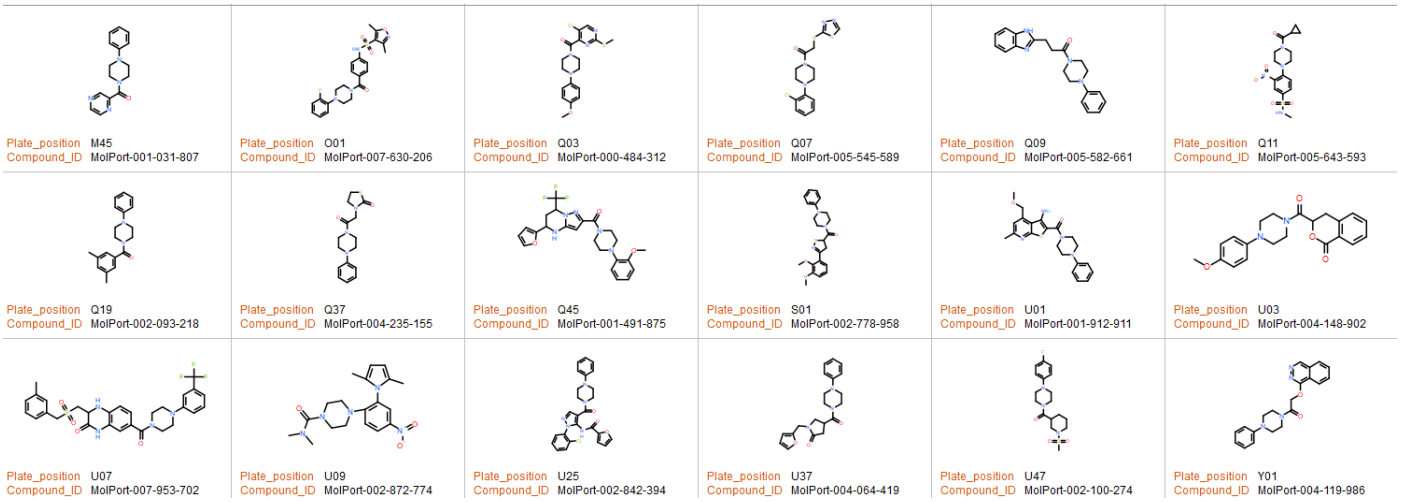
C5	Z394875046	21.1	**F5**	Z55848625	37.0			
C6	Z1509533494	21.0	**F6**	Z1171742721	25.6			

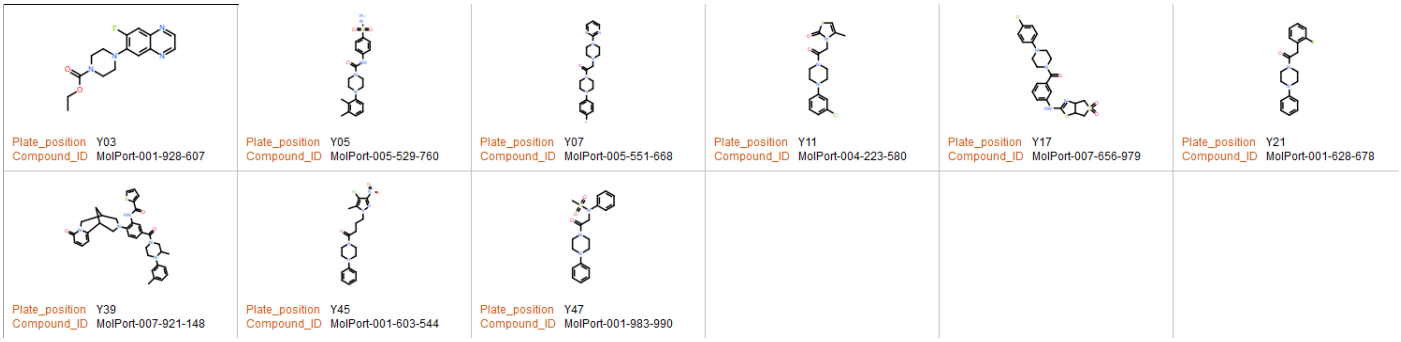
Loop 4 follow-up compounds

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









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 (%)	Plate well	Inhibition at 1 mM (%)	Plate well	Inhibition at 1 mM (%)	Plate well	Inhibition at 1 mM (%)	Plate well	Inhibition at 1 mM (%)
A01	6.1	**AC37**	18.0	**I01**	5.7	**M17**	33.1	**U07**	88.8
A03	24.1	**AC47**	9.3	**I05**	80.6	**M19**	57.4	**U09**	3.4
A05	9.5	**C01**	51.2	**I07**	-3.6	**M37**	76.6	**U25**	17.9
A07	15.8	**E01**	72.0	**I09**	48.4	**M45**	19.7	**U37**	19.3
A09	-5.1	**E03**	19.8	**I11**	3.8	**O01**	4.3	**U47**	2.9
A11	31.4	**E05**	0.8	**I15**	18.2	**Q03**	3.5	**Y01**	13.0
A15	18.7	**E07**	9.9	**I25**	101.8	**Q07**	14.9	**Y03**	21.6
A41	13.9	**E09**	23.3	**I37**	61.8	**Q09**	58.6	**Y05**	63.9
A45	-1.3	**E11**	-2.5	**I43**	13.9	**Q11**	3.2	**Y07**	35.3
AC01	20.3	**E15**	12.3	**I45**	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

Results

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

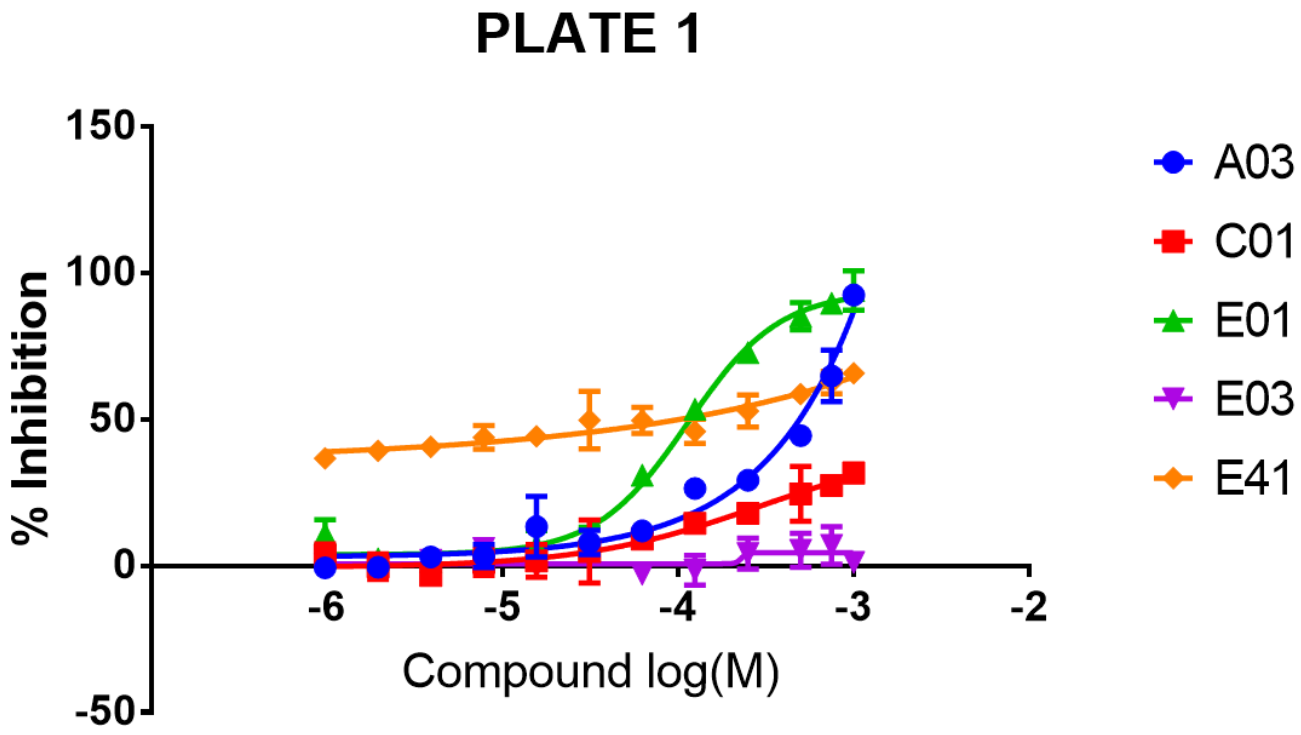
Plate well:	**A03**		**C01**		**E01**		**E03**		**E41**	
Cpd logM	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)
-3.0	92.6	2.2	31.8	1.6	94.2	6.8	1.3	0.7	65.9	1.7
-3.1	65.0	8.8	27.6	3.6	89.8	2.0	7.1	6.4	62.7	3.8
-3.3	44.6	1.2	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

-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 well:	**G01**		**I05**		**I25**		**I37**		**M19**	
Cpd logM	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)
-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
Plate well:	**M37**		**Q09**		**U03**		**U07**		**U37**	
Cpd logM	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)
-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 well:	**Y05**		**Y07**		**Y39**		**Y45**		**AC11**	
Cpd logM	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % 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 logM	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)
-3.0	78.5	8.7	96.2	2.9	61.1	0.8	73.6	1.9	91.3	3.5
-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	10.0	3.7	8.3	5.3	33.5	2.8	-1.6	1.8	19.7	5.3
-6.0	5.2	4.5	6.9	7.1	22.4	5.3	-2.0	1.7	17.6	7.2

Plate well:	**C4**		**D4**		**E2**		**E5**		**F3**	
Cpd logM	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)
-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**	
Cpd logM	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)	Mean % inhibition	SD (%)
-3.0	88.8	5.0	91.9	1.5	90.5	3.2	82.3	3.4	92.3	6.3
-3.1	80.8	2.9	84.8	4.6	81.3	7.8	70.6	0.9	84.5	3.4
-3.3	77.9	1.4	82.5	2.7	71.2	2.2	63.1	3.5	77.5	2.3
-3.6	63.6	1.4	70.9	2.5	62.5	0.4	55.1	3.0	67.6	2.5
-3.9	47.9	4.2	63.5	1.4	56.2	0.9	49.1	0.8	64.3	1.6
-4.2	24.6	7.5	58.2	1.2	52.0	2.9	40.7	0.5	66.1	9.0
-4.5	19.0	1.6	53.8	0.8	49.8	1.0	31.5	1.7	58.3	5.1
-4.8	13.3	1.5	49.8	3.0	49.5	3.9	17.7	0.8	56.5	3.3
-5.1	11.7	1.7	27.4	3.1	49.8	3.9	15.3	1.1	48.7	6.5
-5.4	10.6	2.4	18.0	0.9	43.2	2.6	15.5	1.2	50.8	5.3
-5.7	10.7	4.0	15.5	2.0	36.8	1.9	9.0	2.3	43.2	3.6
-6.0	9.2	4.5	13.6	2.2	32.5	2.0	7.4	3.0	40.0	1.6

GraphPad plots



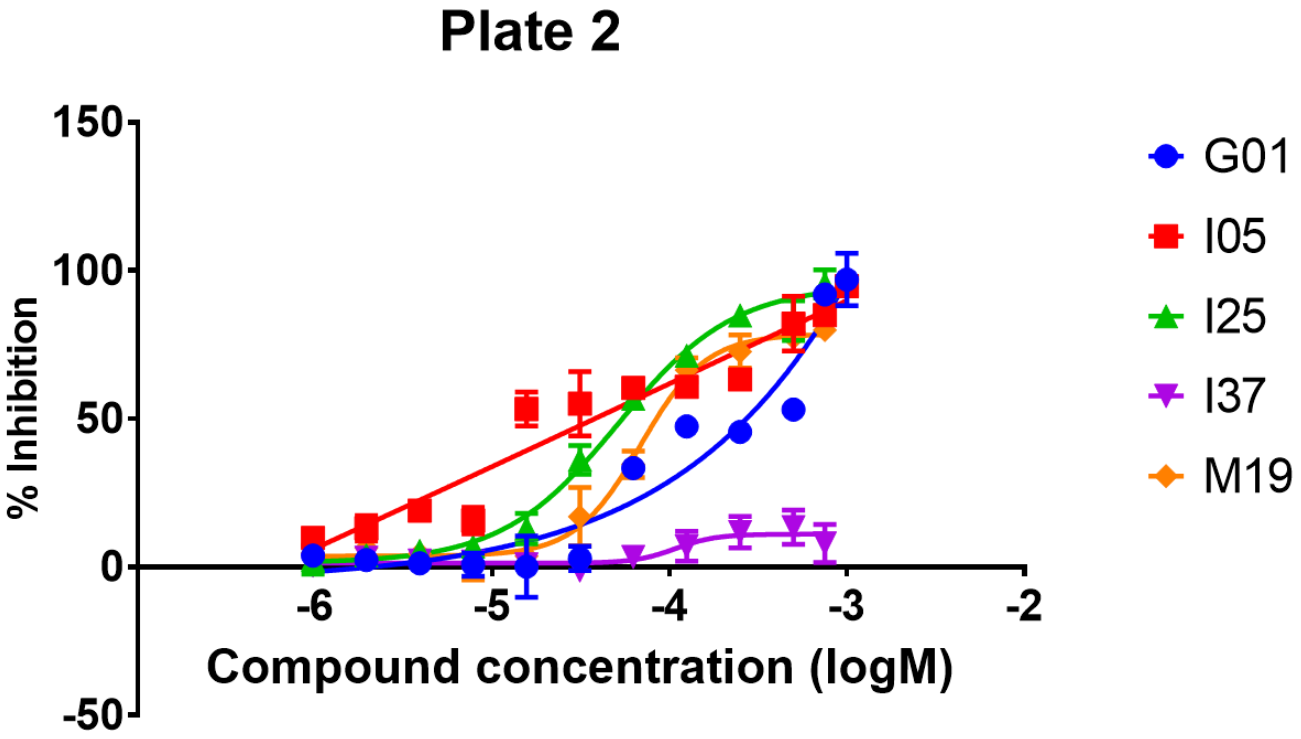


Plate 3

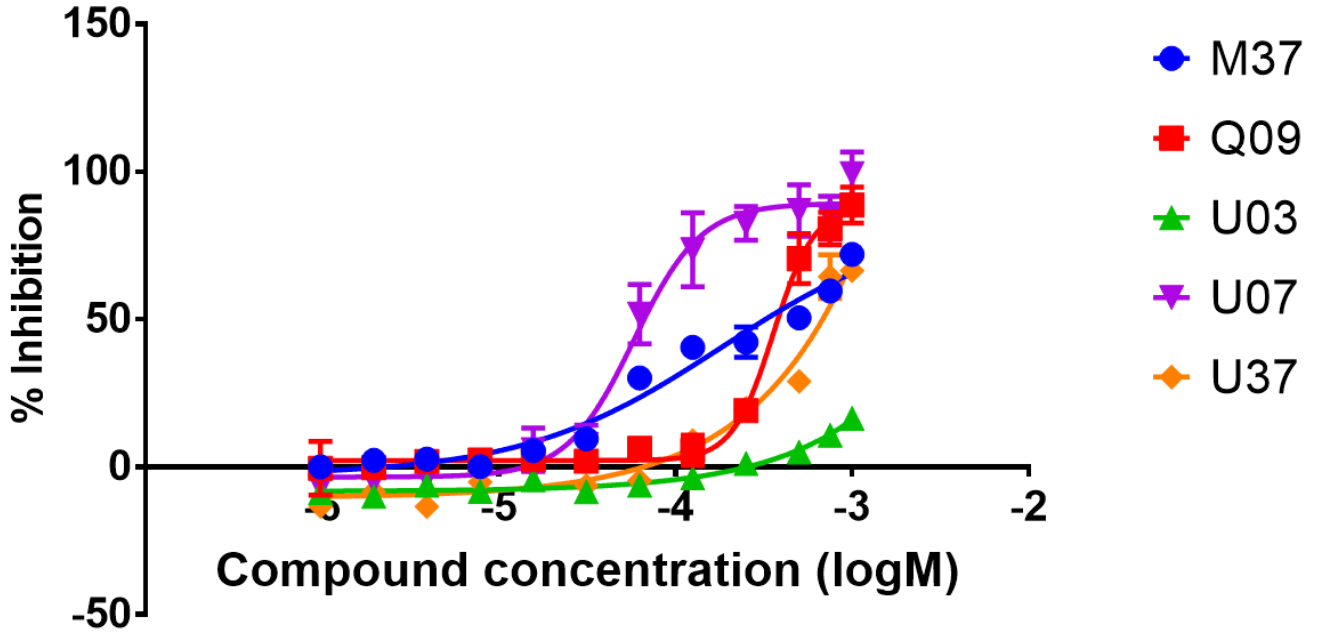


Plate 4

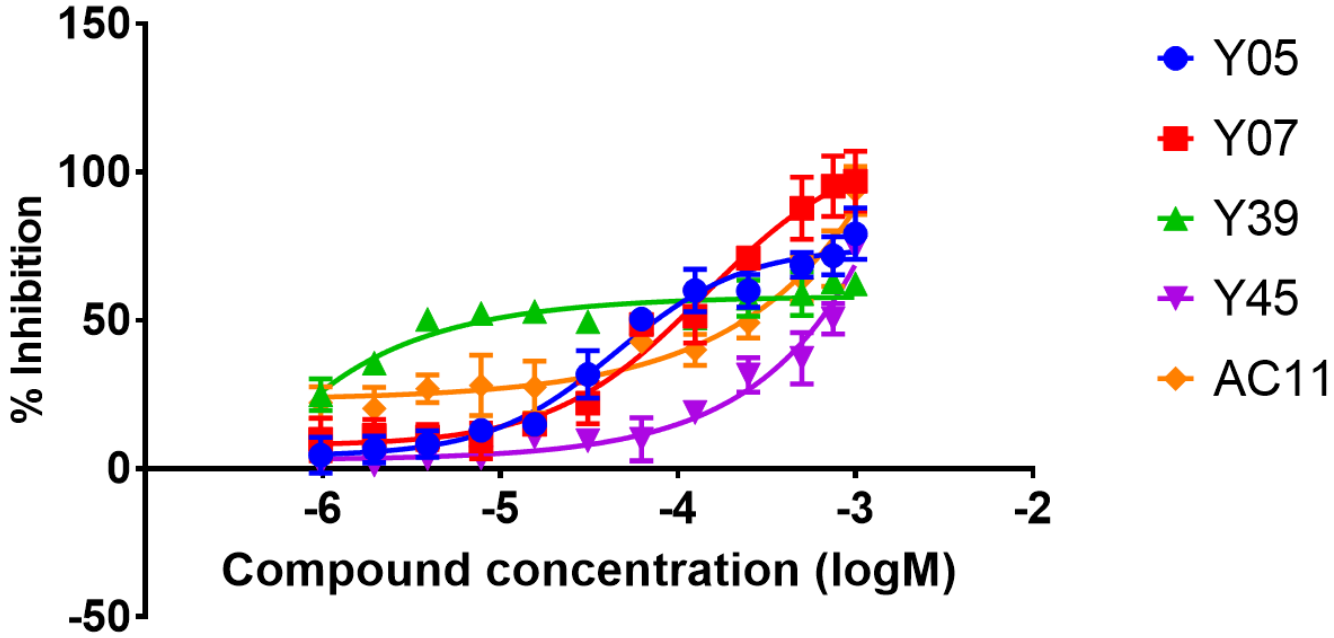


Plate 5

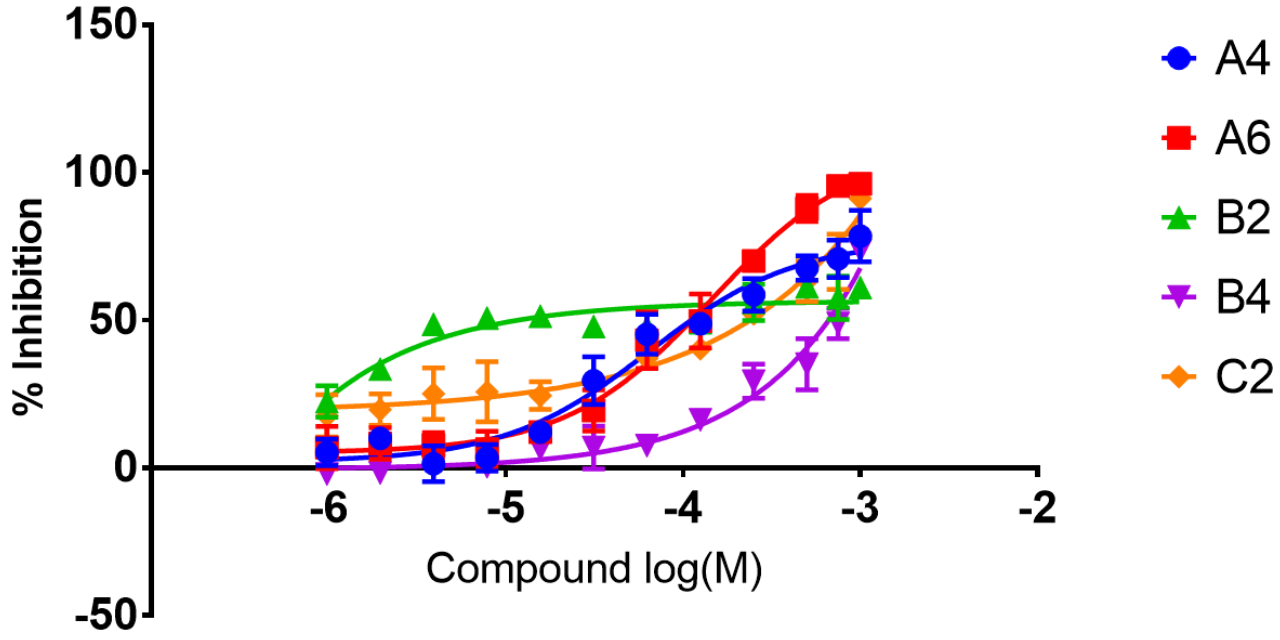
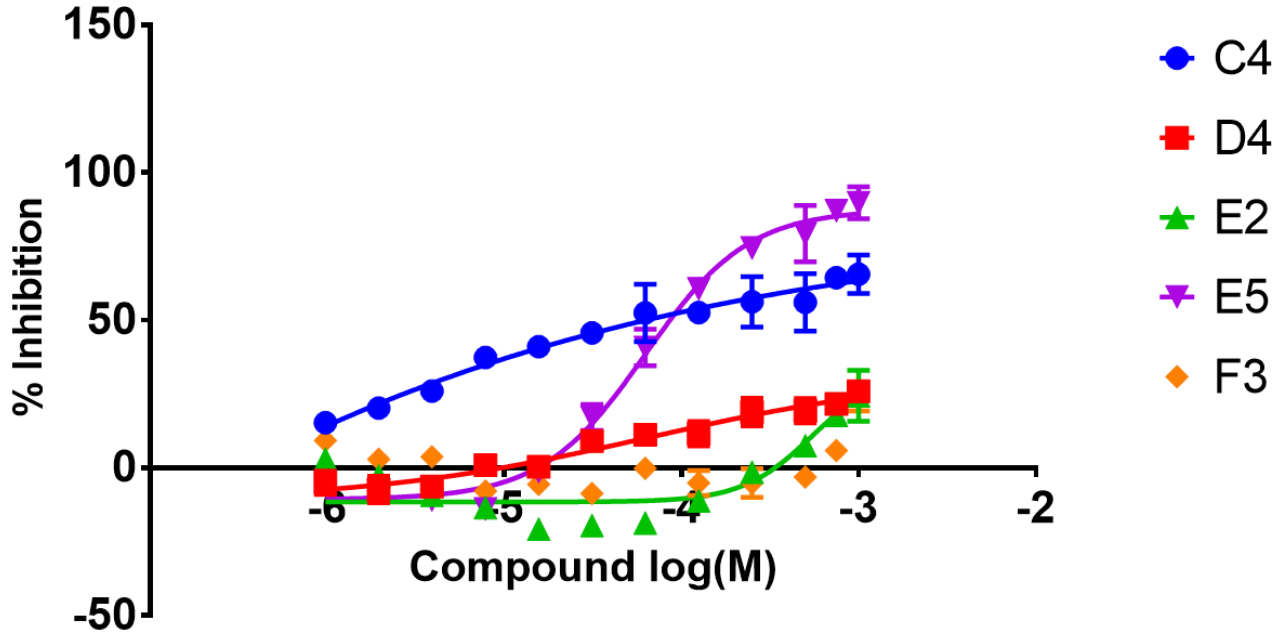
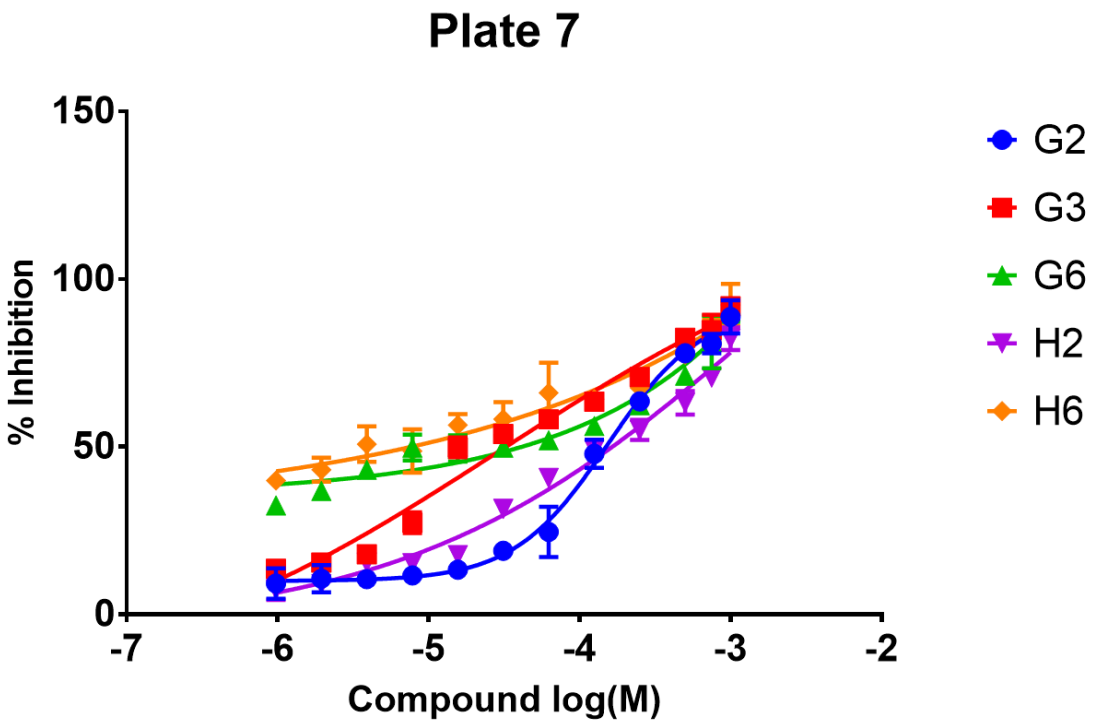


Plate 6

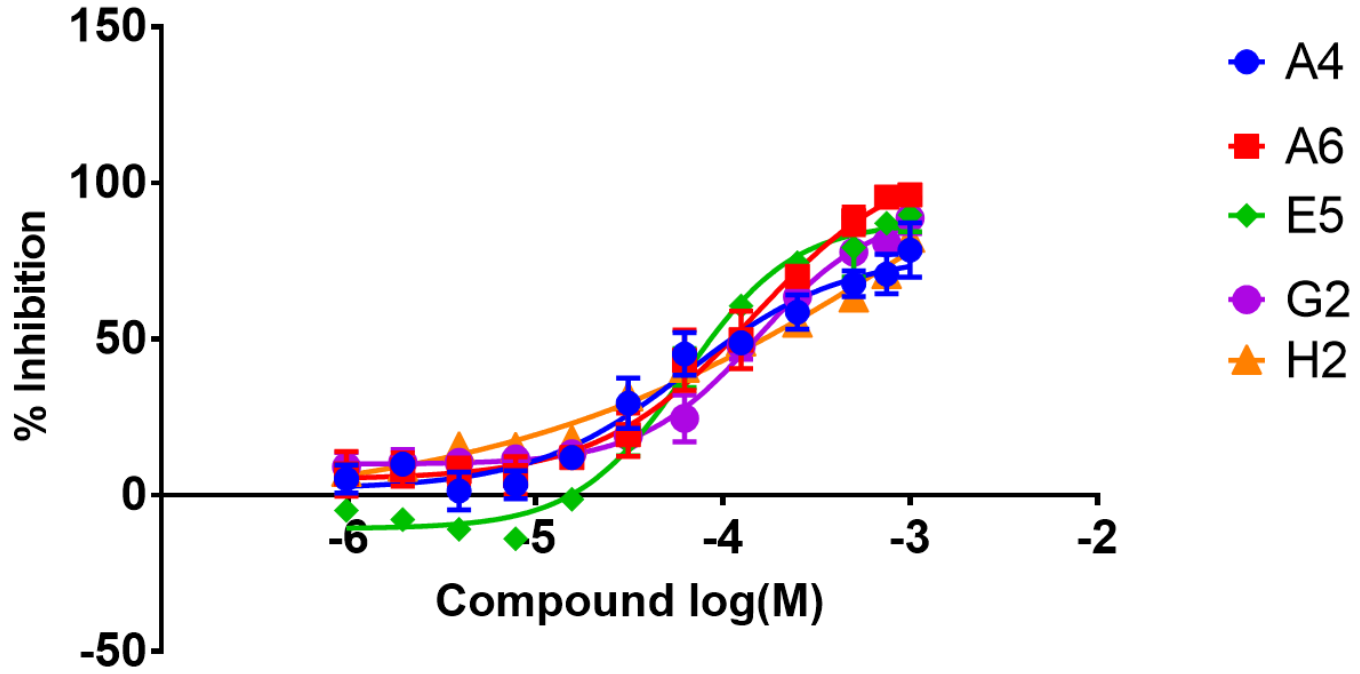


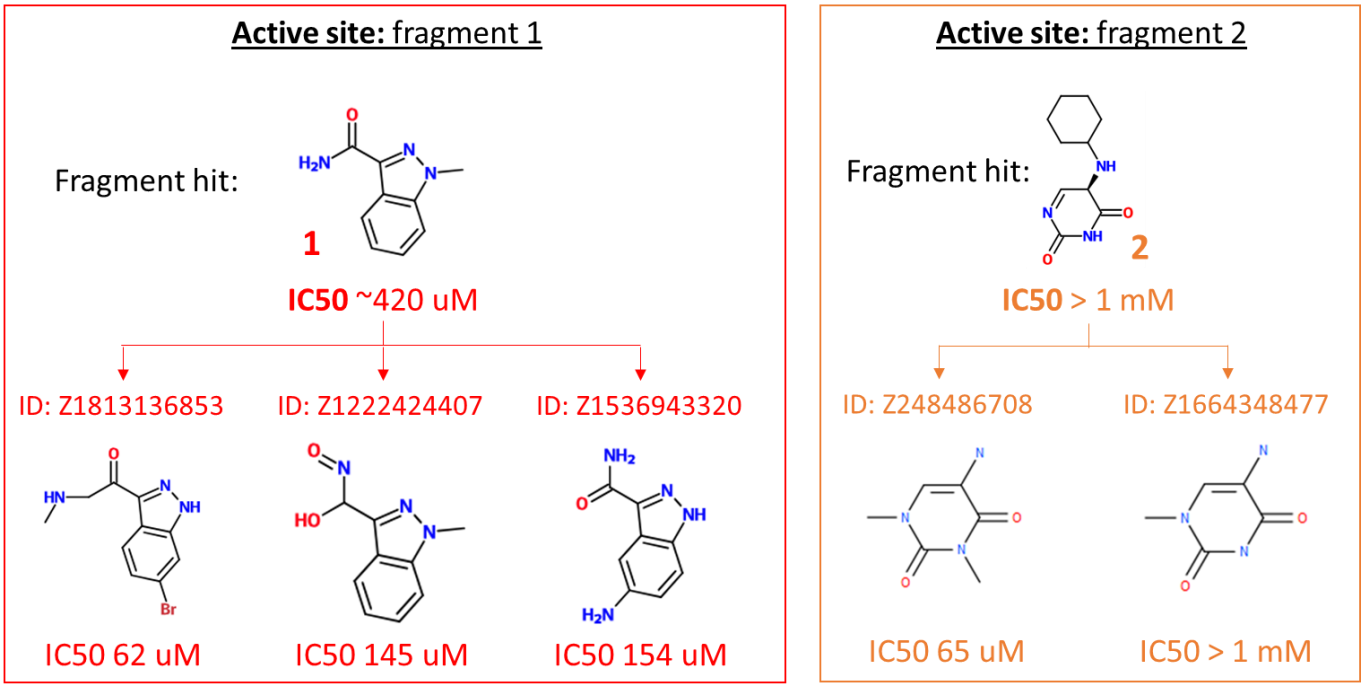


Analysis

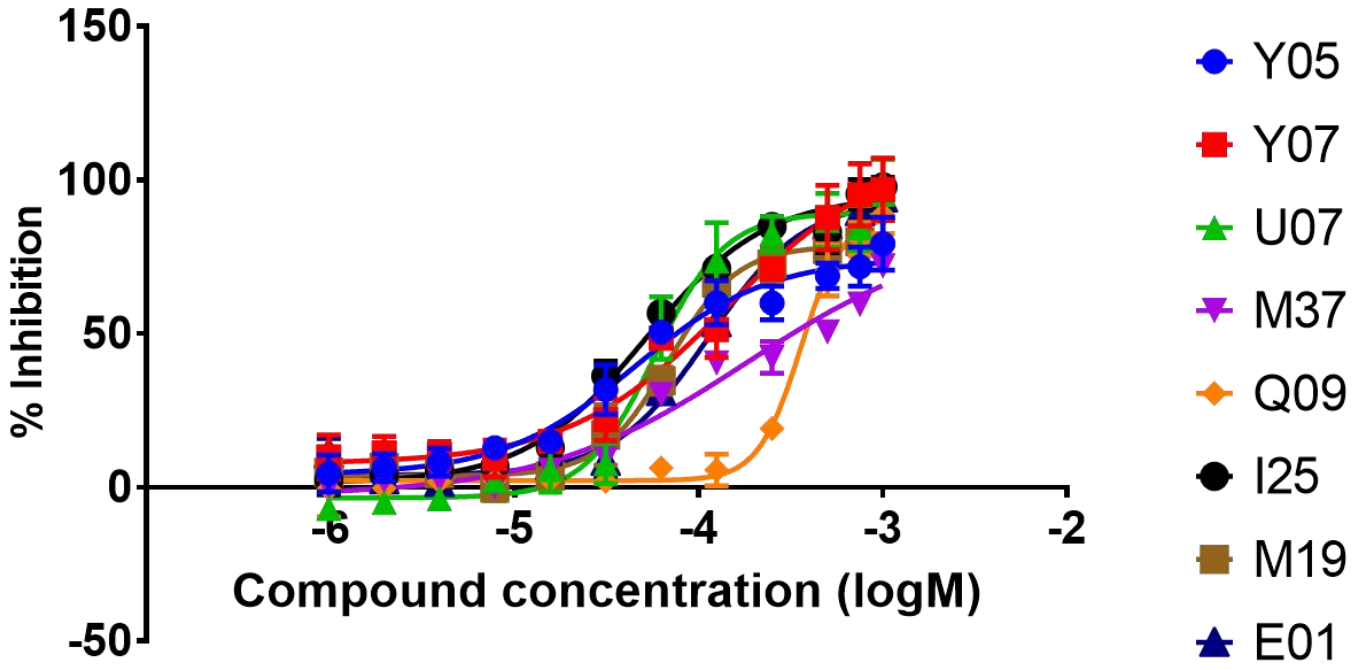
Total of 5 active site follow-up compounds and 8 gating loop follow-up compounds showed non-ambiguous curve fitting and high enough inhibition of HAO1 activity to allow IC50 determination. The appropriate fitted curves and calculated IC50 values are shown below.

Well-fitted curves for active site FUPs:



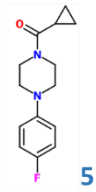


Well-fitted curves for loop FUPs:



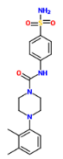
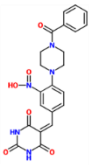
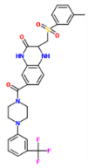
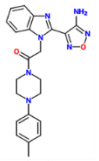
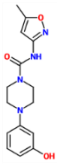
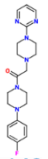
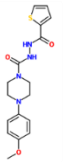
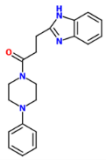
Gating loop: fragment 5

Fragment hit:



5

IC50 >> 1 mM

ID: BCC0013909	ID: BCC0040031	ID: BCC0042757	ID: BCC0089054	ID: BCC0118038	ID: BCC0026181	ID: BCC0123306	ID: BCC0016684
							
IC50 45 uM	IC50 51 uM	IC50 58 uM	IC50 69 uM	IC50 114 uM	IC50 142 uM	IC50 182 uM	IC50 359 uM