SUPPORTING INFORMATION

Structure-based optimization of ML300 derived, non-covalent inhibitors targeting the severe acute respiratory syndrome coronavirus 3CL protease (SARS-CoV-2 3CL^{pro})

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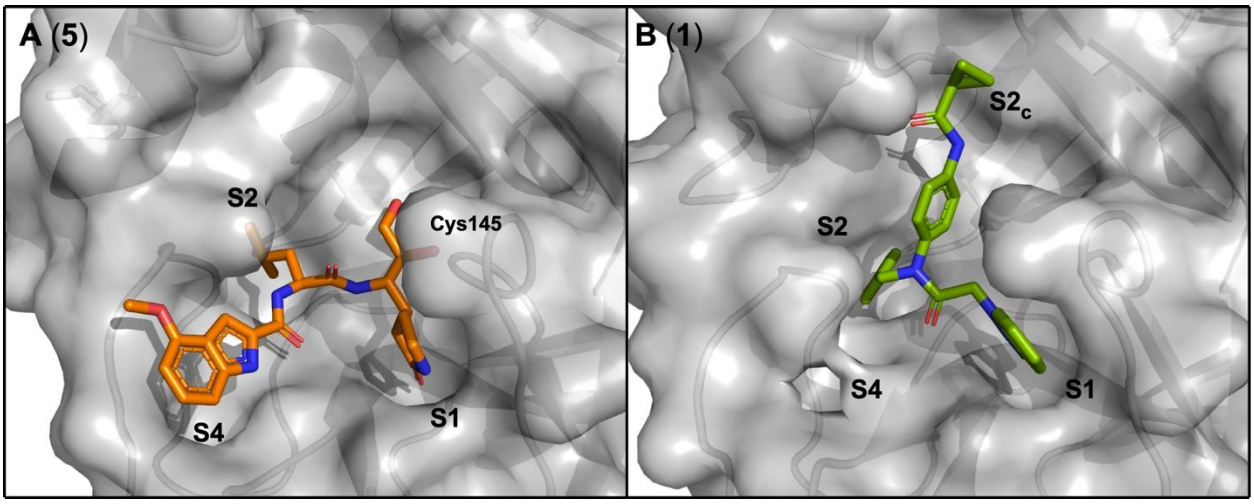
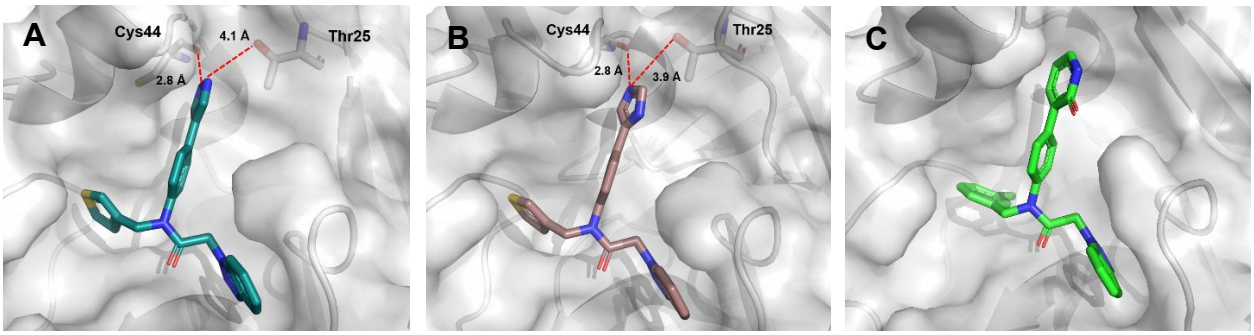


Supplemental Figure S1. Comparison of 3CL^{pro} main binding site when bound to covalent inhibitor **5**, and this series lead, **1**. The two molecules have significantly different binding poses, occupying alternate regions of the enzyme binding pocket. **A** – Published X-ray co-crystal structure of **5** – SARS-CoV-2 3CL^{pro} (PDB: 6XHM); **B** – X-ray co-crystal structure of **1** (ML300) bound to SARS-CoV-2 3CL^{pro} (PDB: 7LME).



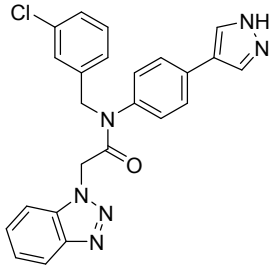
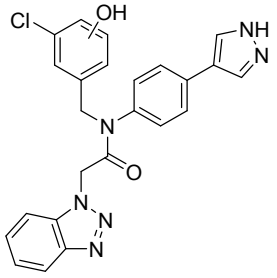
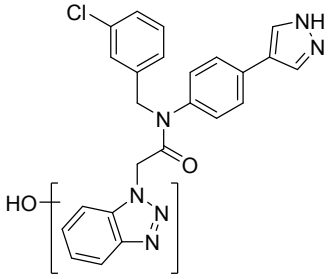
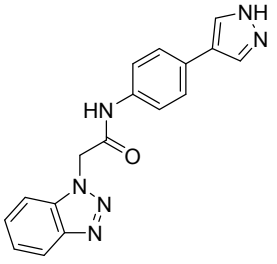
Supplemental Figure S2. X-ray crystal structures of SARS-CoV-1 3CL^{pro} in complex with compounds, **19** (A), **21** (B), and **35** (C). All structures were generated by soaking apo-crystals with compound DMSO stock, and via this methodology we observe a glycerol molecule (not shown) in each structure in the S4 region.

Supplemental Table S1. Summary of tier 1 DMPK data.

Cmpd.	HLM[a]		Human S9		RLM[b]		PPB, F_u (%)[c]		MDCK[d]	CYP Profile (% Inh. at 10 µM)[e]		
	$t_{1/2}$ (min)	$CL_{int'}$ (mL/min/kg)	$CL_{int'}$ (mL/min/kg)	CL_{hep} (mL/min/kg)	$t_{1/2}$ (min)	$CL_{int'}$ (mL/min/kg)	Human Plasma	Rat Plasma	P_{app} [A-B] (x10-6 cm/s)	3A4	2D6	2C9
8	2.0	1263.5			*	-	0.62%	**	80	85.7	44.8	63.4
19	1.7	1465.1			2.6	1995	0.35%	**	59.8	95.8	61.9	98.0
21	16.8	168			10.0	553	1.7%	**	33	98.8	80	99
25	1.1	2378.4			*	-	0.25%	2.20%	81	89.6	45.5	72.3
27	19.1	125.3			2.2	2568	2.41%	23.00%	71.6	64.0	18.3	1.7
28	*	-			*	-	0.20%	0.74%	55	92.8	79.5	89.5
31	*	-			*	-	0.24%	0.75%	59.5	93.3	82.9	89.2
33	*	-			*	-	0.07%	0.29%	24	95.6	82.0	84.7
34	2.6	1080			*	-	0.8%	5.3%	66.9	95.4	67	59
35	4.4	574.9			1.6	3461	1.42%	0.48%	43.8	69.6	22.6	75.0
36	1.0	2621	436	20	1.0	5668	0.1%	**	39.4	96	63	99
38	1.0	2496			1.0	5525	<0.06%	0.8%	20.9	97.1	69	100
39	1.2	2036			*	-	0.9%	6.1%	46.7	92.0	44	89
40	1.2	2175.2	471	20.1	*	-	0.2%	5.6%	61.8	94.1	42	97
41	21.1	141			12.3	589	0.7%	1.8%	46.1	100	87.2	100
42	2.5	980.7	256	19.4	1.6	3555	0.4%	3.5%	61.8	99	55	98.5
43	4.1	620.4	145	18.3	1.7	3302	1.8%	11.0%	66.7	87.7	25.7	91.9

Tier 1 DMPK generated at Q2 solutions, except hS9 data generated at Lipscomb University College of Pharmacy. [a] HLM – Human liver microsomes; [b] RLM – Rat liver microsomes; [c] F_u – Fraction Unbound; [d] Permeability in MDCK-MDR1 cells; [e] CYP % inhibition at 10 µM; * – Rapid clearance, not reliably measured; ** – Low compound recovery

Supplemental Table S2. Fragmentation of compound **36** and proposed metabolites in Human S9.

Metabolite	Structure	$[M+H]^+$	Fragment Ions
36 Parent		443	326, 134, 127, 118
M1 [a]60% of total metabolite peak area		459	301, 202, 174, 143
M2 33% of total metabolite peak area		459	326, 151, 127
M3 7% of total metabolite peak area		319	202, 186, 174, 119

[a] Metabolite formation (%) based on the total peak area (total peak AUC) of the three principle metabolites, M1, M2 and M3.

Structural Biology Experimental Details

Supplemental Table S3. SARS-CoV-2 3CL^{pro} X-ray refinement statistics. One crystal was used for each structure. Values in parentheses unless stated represent data in the highest-resolution shell. RMS = Root mean square

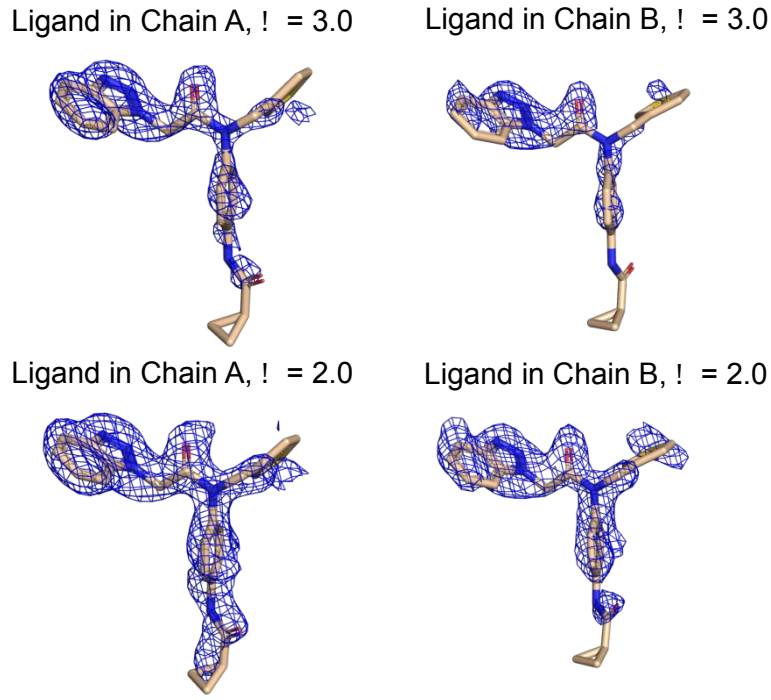
Compound	**1**	**19**	**21**
PDB Accession Code	**7LME**	**7LMD**	**7LMF**
Data Collection			
Beamline	APS 21-ID-F	APS 21-ID-F	APS 21-ID-F
Wavelength (Å)	0.978	0.978	0.978
Resolution range (Å)	43.72–2.10 (2.18–2.10)	24.49–1.96 (2.03–1.96)	43.75–2.20 (2.28–2.20)
Space group	P 1 21 1	C 1 2 1	P 1 21 1
Unit cell			
a, b, c (Å)	44.45, 53.75, 114.18	115.16, 54.34, 44.45	44.54, 53.70, 114.75
α, β, γ (°)	90, 101.98, 90	90, 101.01, 90	90, 100.80, 90
Total reflections	227385	61969	204060
Unique reflections	30873 (3016)	19327 (1911)	27231 (2691)
Multiplicity	7.4 (5.9)	3.2 (3.0)	7.5 (6.9)
Completeness (%)	99.16 (94.27)	98.68 (98.25)	99.53 (96.93)
Mean I/σI	15.57 (1.95)	31.63 (6.77)	19.00 (2.37)
Wilson B-factor	26.18	23.99	29.82
R-merge	0.139 (0.831)	0.066 (0.237)	0.126 (0.793)
R-meas	0.149 (0.910)	0.079 (0.288)	0.135 (0.857)
R-pim	0.054 (0.366)	0.043 (0.162)	0.049 (0.322)
CC1/2	0.996 (0.705)	0.994 (0.901)	0.995 (0.793)
Structure Refinement			
Reflections used in refinement	30823 (2928)	19326 (1911)	27179 (2623)
Reflections used for R-free	1481 (143)	960 (78)	1384 (116)
R-work	0.1802 (0.2292)	0.1878 (0.2261)	0.1857 (0.2307)
R-free	0.2405 (0.2870)	0.2340 (0.2919)	0.2539 (0.3330)
Number of non-hydrogen atoms	4975	2580	4912
macromolecules	4673	2333	4704
ligands	62	30	60
solvent	240	217	148
Protein residues	609	302	610
RMS (bonds)	0.007	0.007	0.007
RMS (angles)	0.84	0.89	0.91
Ramachandran favored (%)	98.35	98.00	96.20
Ramachandran allowed (%)	1.49	2.00	3.30
Ramachandran outliers (%)	0.17	0	0.50
Rotamer outliers (%)	0	0	0
Clash score	3.68	3.66	3.95
Average B-factor	28.60	27.08	33.22
macromolecules	28.33	26.70	33.10
ligands	36.2	30.74	40.36
solvent	31.8	30.63	34.2

Supplemental Table S4. SARS-CoV-1 3CL^{pro} X-ray refinement statistics. One crystal was used for each structure. Values in parentheses unless stated represent data in the highest-resolution shell. RMS = Root mean square

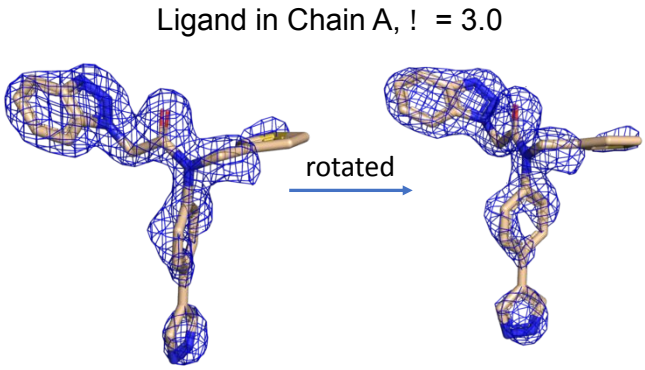
Compound	**8**	**19**	**21**	**35**
PDB Accession Code	**7LMH**	**7LMI**	**7LMG**	**7LMJ**
Data Collection				
Beamline	APS 21-ID-F	APS 21-ID-F	APS 21-ID-F	APS 21-ID-F
Wavelength (Å)	0.978	0.978	0.978	0.978
Resolution range (Å)	32.30 - 1.85 (1.92 - 1.85)	32.34 - 1.71 (1.77 - 1.71)	32.33 - 1.69 (1.75 - 1.69)	32.06 - 1.60 (1.66 - 1.60)
Space group	C 1 2 1	C 1 2 1	C 1 2 1	C 1 2 1
Unit cell				
a, b, c (Å)	107.69 82.47 53.30	108.51 82.11 53.38	107.54 82.71 53.20	108.41 81.82 53.36
α, β, γ (°)	90 105.2 90	90 104.7 90	90 105.3 90	90 104.7 90
Total reflections	76397	97473	99960	114896
Unique reflections	38216 (3632)	48839 (4776)	50018 (4788)	57645 (5434)
Multiplicity	7.6 (7.4)	7.6 (7.4)	7.6 (7.4)	7.6 (7.4)
Completeness (%)	99.42 (94.90)	99.75 (98.09)	99.50 (95.68)	96.98 (90.93)
Mean I/σI	22.31 (7.90)	10.76 (2.59)	17.06 (2.73)	7.38 (3.20)
Wilson B-factor	32.29	28.83	32.53	23.62
R-merge	0.019 (0.092)	0.055 (0.322)	0.030 (0.242)	0.085 (0.277)
R-meas	0.027 (0.130)	0.077 (0.455)	0.042 (0.342)	0.120 (0.392)
R-pim	0.019 (0.092)	0.055 (0.322)	0.030 (0.242)	0.085 (0.277)
CC1/2	0.999 (0.985)	0.987 (0.834)	0.995 (0.923)	0.99 (0.97)
Structure Refinement				
Reflections used in refinement	38200 (3632)	48829 (4773)	50015 (4788)	57604 (5426)
Reflections used for R-free	1903 (165)	2448 (269)	2502 (253)	2896 (299)
R-work	0.1540 (0.1797)	0.1957 (0.3025)	0.1928 (0.2935)	0.1968 (0.2418)
R-free	0.2004 (0.2878)	0.2094 (0.3297)	0.2216 (0.2963)	0.2345 (0.2909)
Number of non-hydrogen atoms	2709	2675	2757	2567
macromolecules	2393	2420	2390	2401
ligands	43	42	66	36
solvent	273	213	301	130
Protein residues	306	306	306	306
RMS (bonds)	0.018	0.016	0.015	0.019
RMS (angles)	2.13	2.03	1.96	2.17
Ramachandran favored (%)	97.37	97.37	98.68	97.37
Ramachandran allowed (%)	2.63	2.63	1.32	2.63
Ramachandran outliers (%)	0	0	0	0
Rotamer outliers (%)	3.76	2.95	1.13	2.62
Clash score	3.96	4.31	13.01	2.29
Average B-factor	38.61	34.62	41.22	28.53
macromolecules	37.43	33.83	39.04	28.33
ligands	43.22	39.88	45.30	28.18
solvent	48.22	42.60	57.70	32.34

Supplemental Figure S3. SARS-CoV-1 and CoV-2 3CL^{pro} F_o-F_c likelihood-weighed omit maps for ligands, with respective sigma contours given:

A: SARS-CoV-2 3CL^{pro} in complex with compound **1** (PDB:7LME)

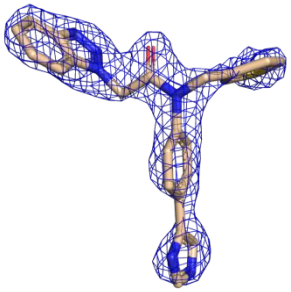


B: SARS-CoV-2 3CL^{pro} in complex with compound **19** (PDB:7LMD)



C: SARS-CoV-2 3CL^{pro} in complex with compound **21** (PDB: 7LMF)

Ligand in Chain A, ! = 3.0



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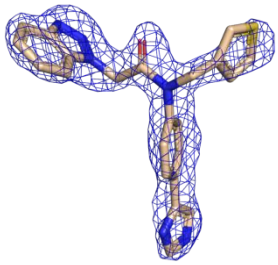

D: SARS-CoV-1 3CL^{pro} in complex with compound **8** (PDB: 7LMH)

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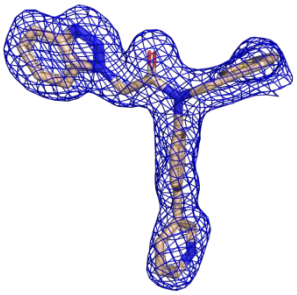

E: SARS-CoV-1 3CL^{pro} in complex with compound **19** (PDB: 7LMI)

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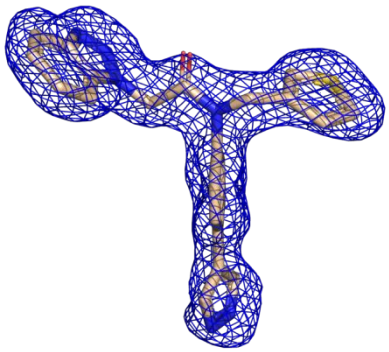
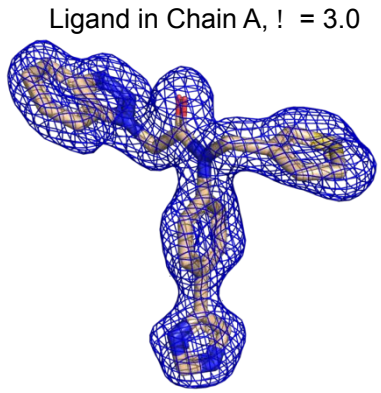
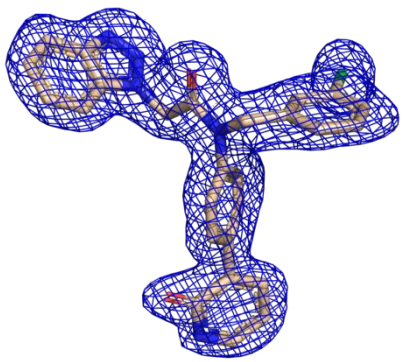


F: SARS-CoV-1 3CL^{pro} in complex with compound **21** (PDB: 7LMG)

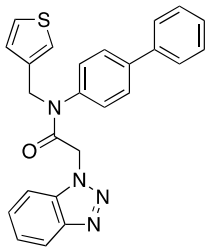


G: SARS-CoV-1 3CL^{pro} in complex with compound **35** (PDB: 7LMJ)



Compound HPLC Traces

LCMS Method A (see main manuscript) – shown are UV traces @ 254 nm



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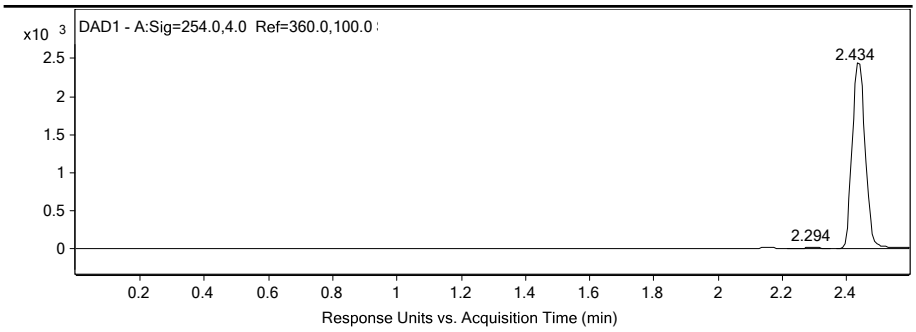

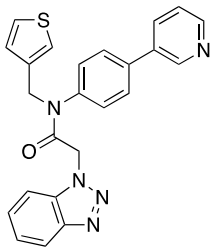

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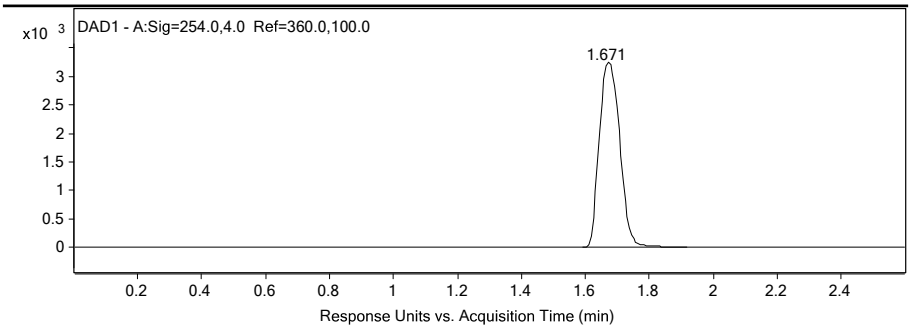

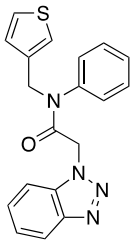

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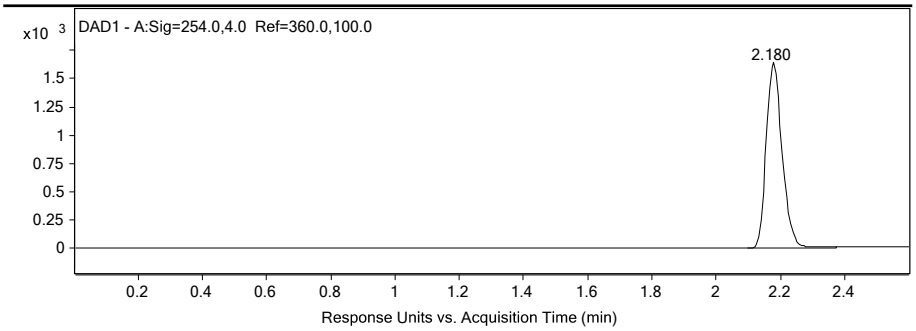

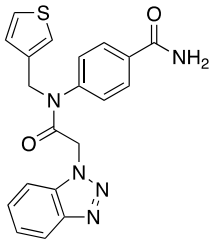

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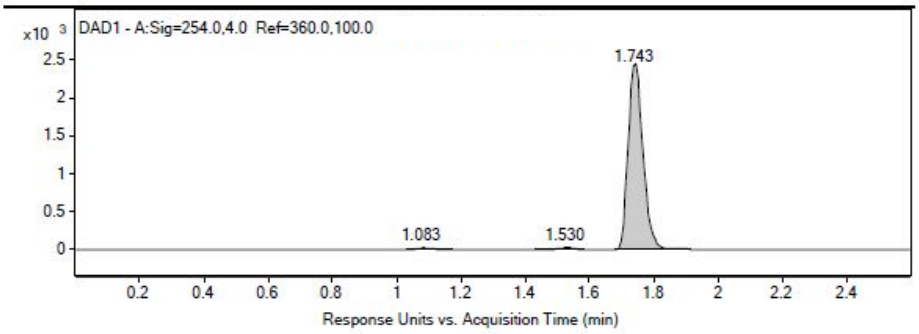

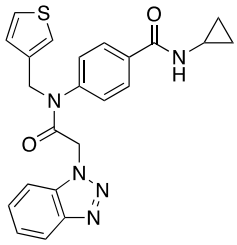

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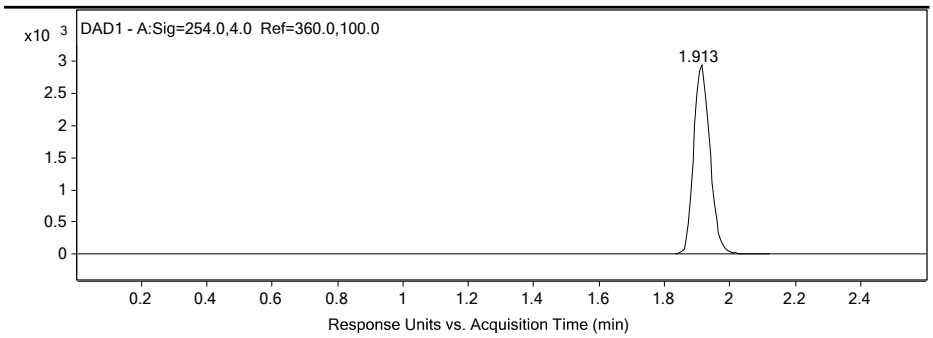
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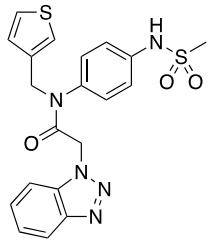

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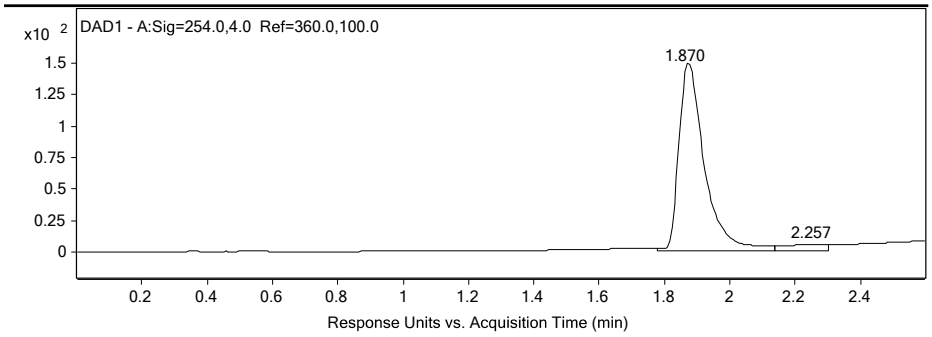
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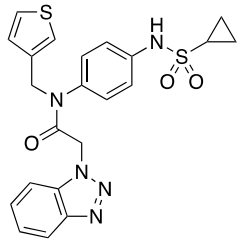
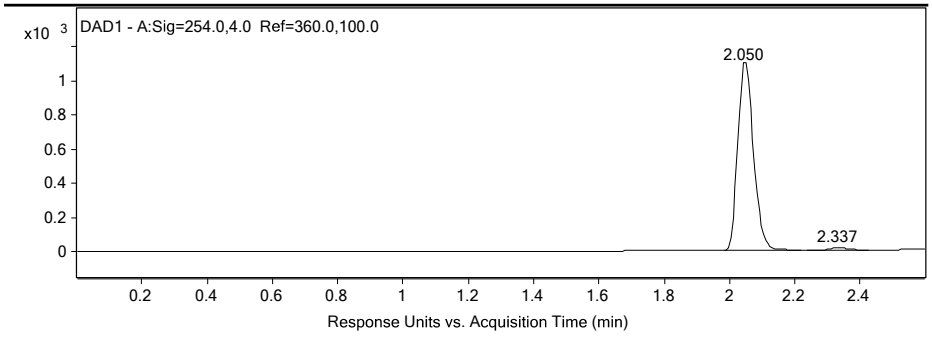
2.257
Response Units vs. Acquisition Time (min)



13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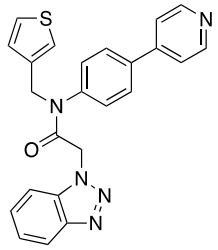
x10 3
2.050
2.337
Response Units vs. Acquisition Time (min)



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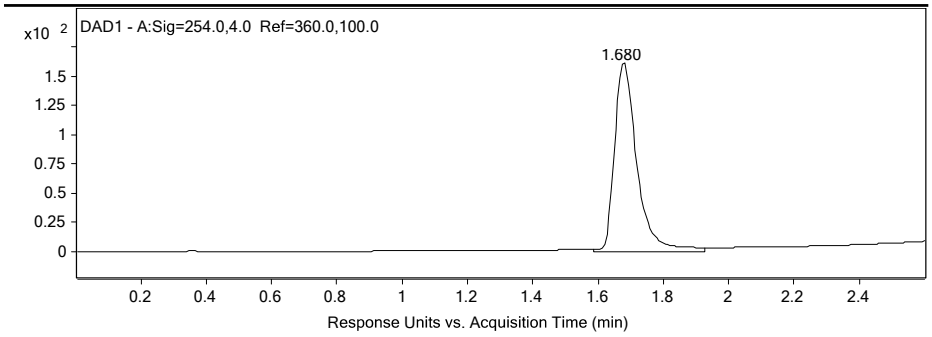
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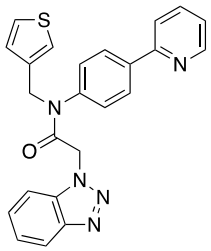
x10 2
1.680
Response Units vs. Acquisition Time (min)



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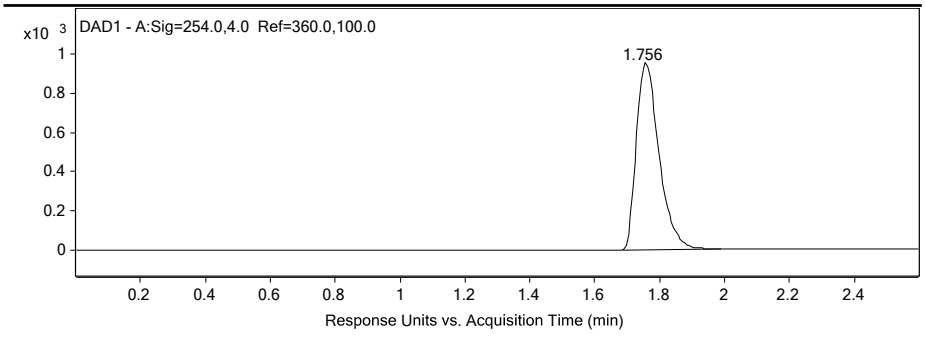
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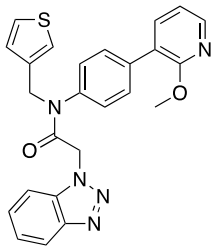
x10 3
1.756
Response Units vs. Acquisition Time (min)



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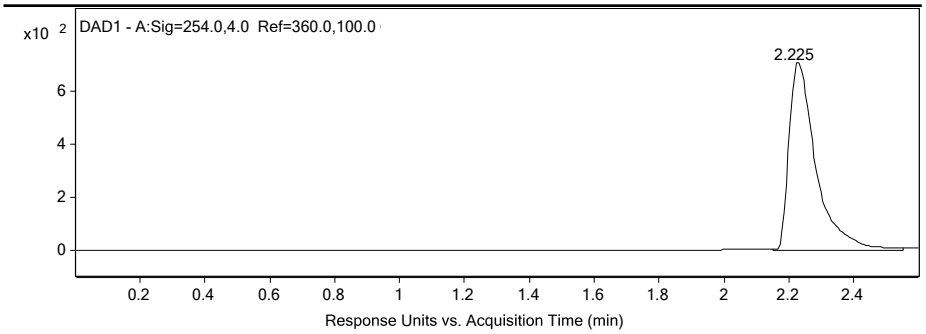
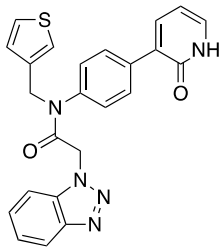

x10 2
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
2.225
Response Units vs. Acquisition Time (min)



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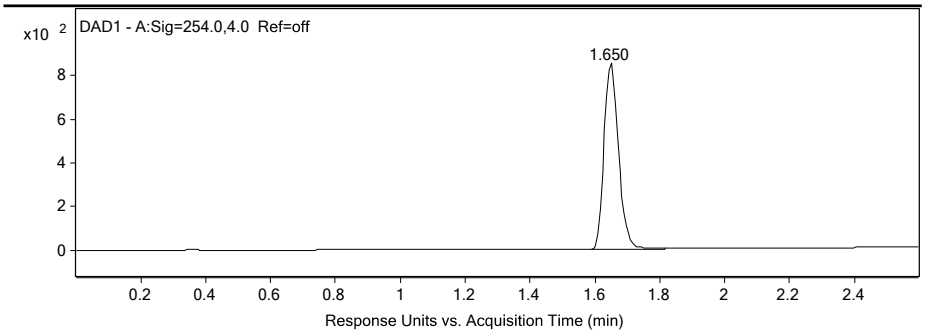
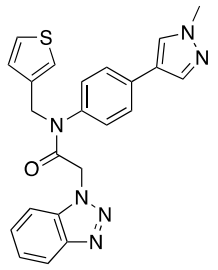

x10 2
DAD1 - A:Sig=254.0,4.0 Ref=off
1.650
Response Units vs. Acquisition Time (min)



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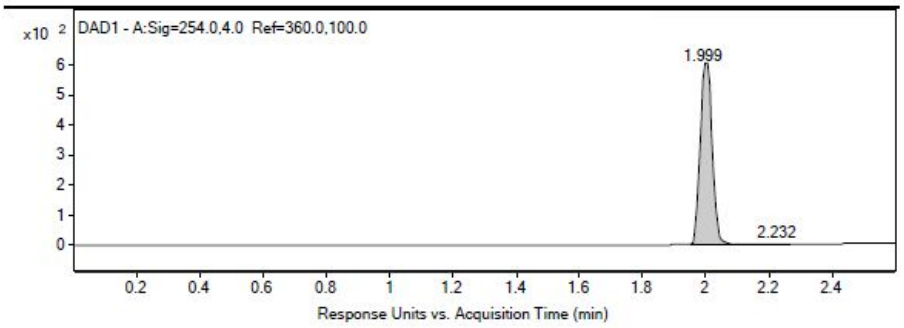
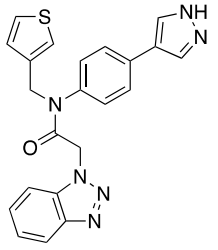

x10 2
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
1.999
2.232
Response Units vs. Acquisition Time (min)



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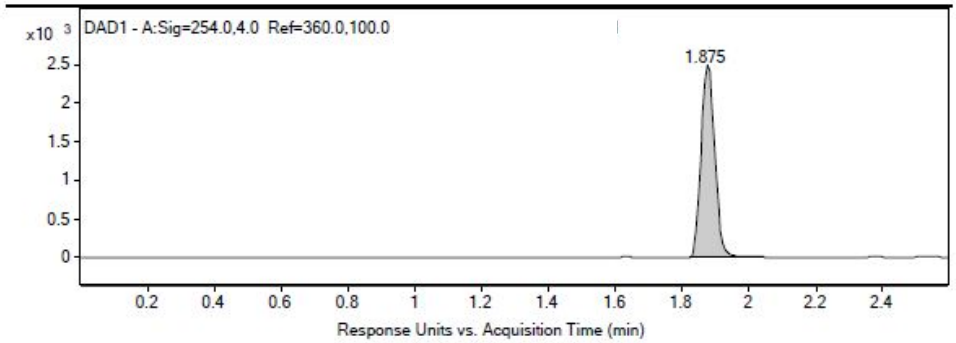
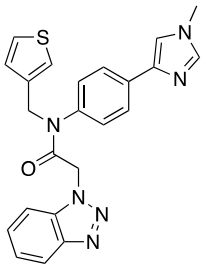

x10 3
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
1.875
Response Units vs. Acquisition Time (min)



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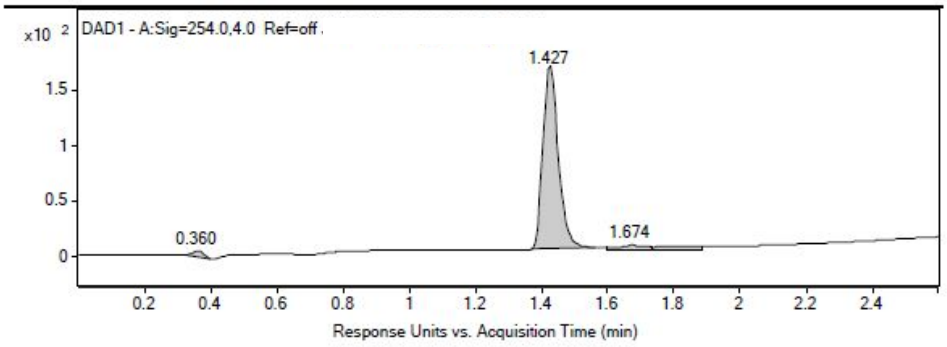
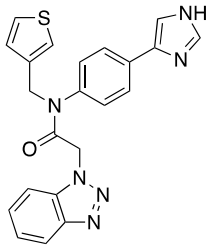

x10 2
DAD1 - A:Sig=254.0,4.0 Ref=off
1.427
0.360
1.674
Response Units vs. Acquisition Time (min)



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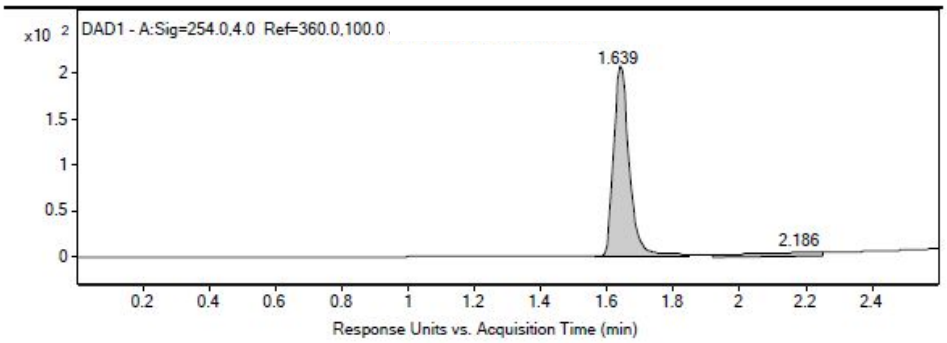
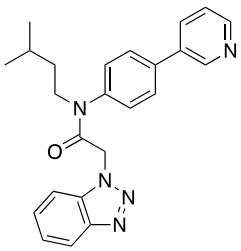

x10 2
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
1.639
2.186
Response Units vs. Acquisition Time (min)



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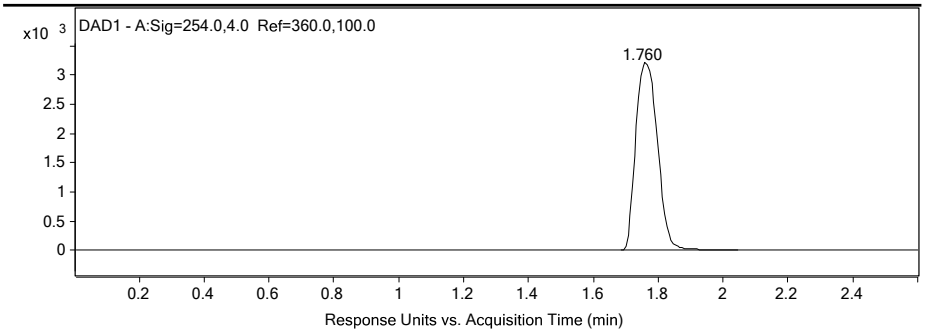
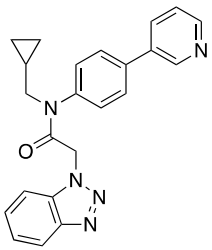

x10 3
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
1.760
Response Units vs. Acquisition Time (min)



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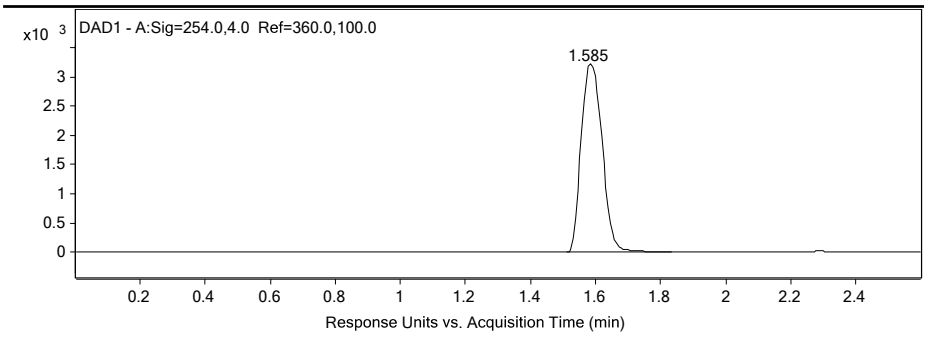
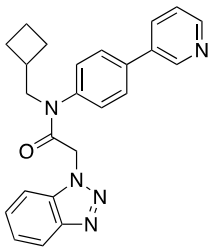

x10 3
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
1.585
Response Units vs. Acquisition Time (min)



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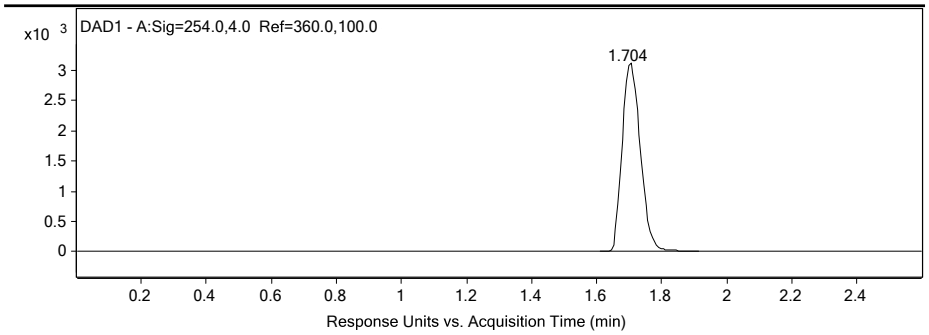
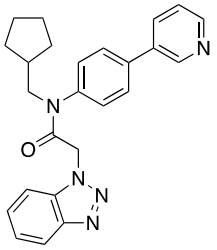

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 3
1.704
Response Units vs. Acquisition Time (min)



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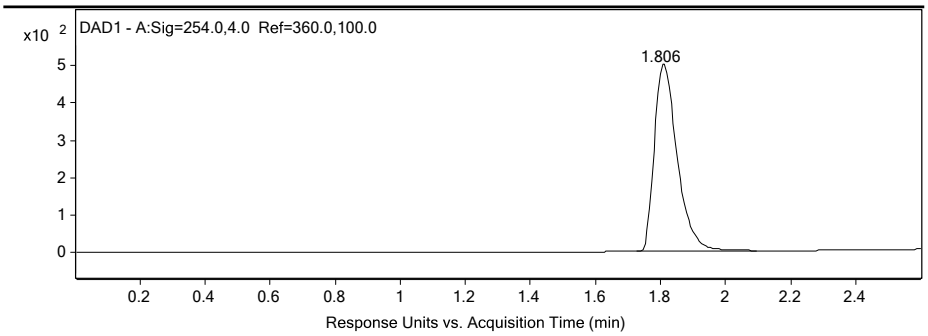
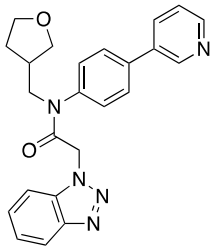
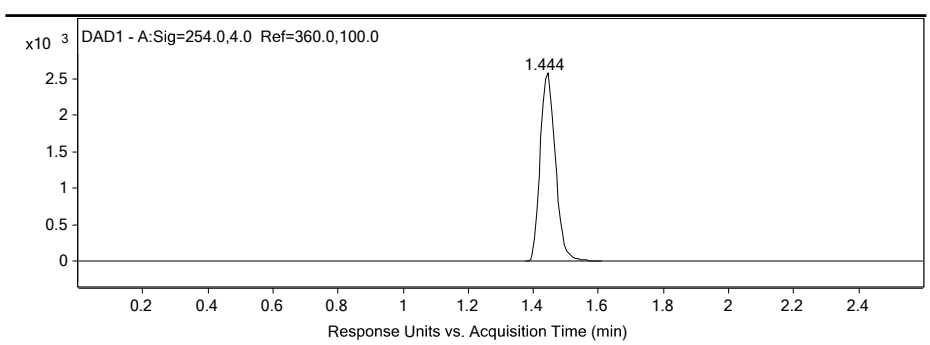
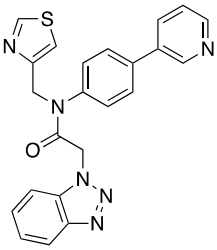

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 2
1.806
Response Units vs. Acquisition Time (min)



26


DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 3
1.444
Response Units vs. Acquisition Time (min)



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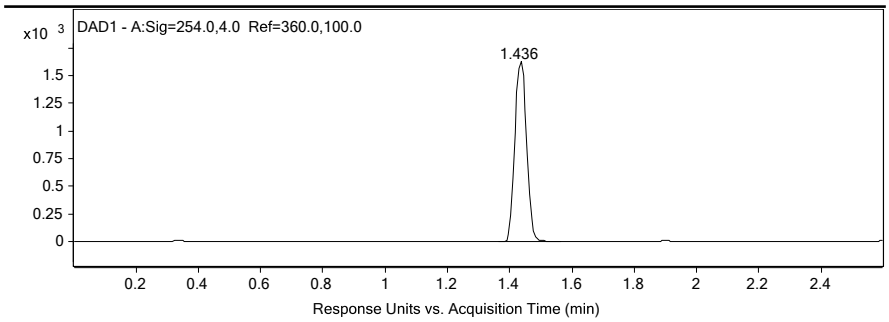
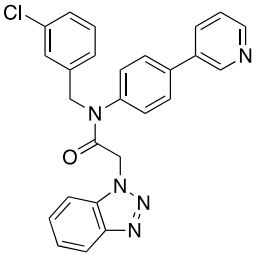
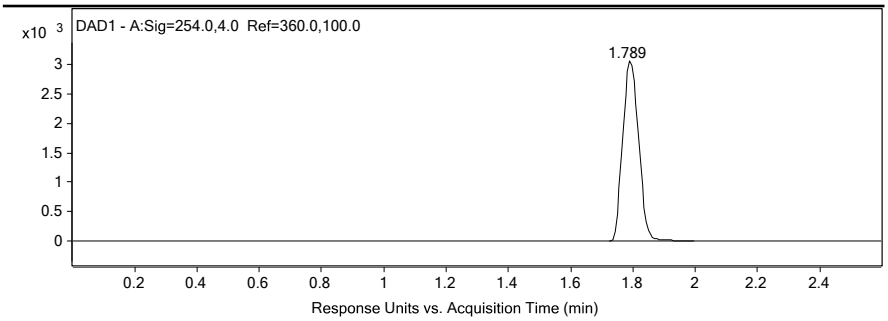
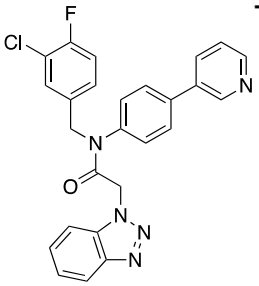

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 3
1.436
Response Units vs. Acquisition Time (min)



28


DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 3
1.789
Response Units vs. Acquisition Time (min)



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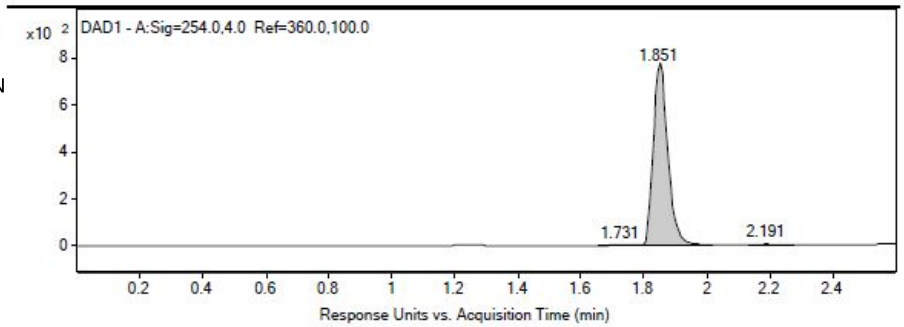
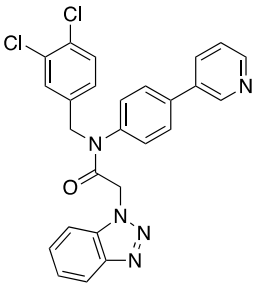

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 2
1.851
1.731
2.191
Response Units vs. Acquisition Time (min)



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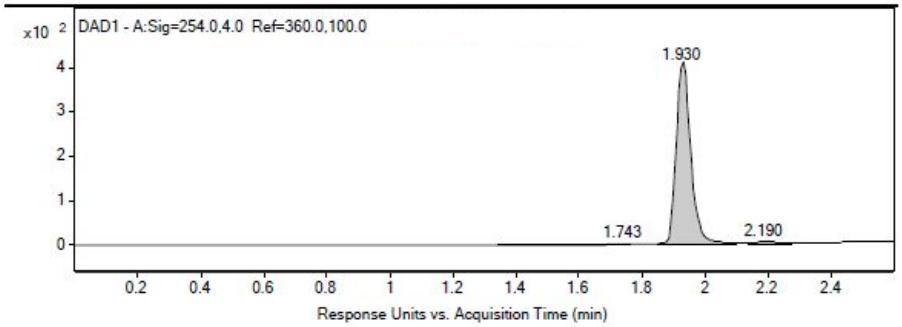
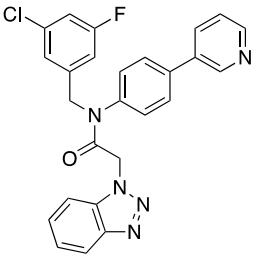
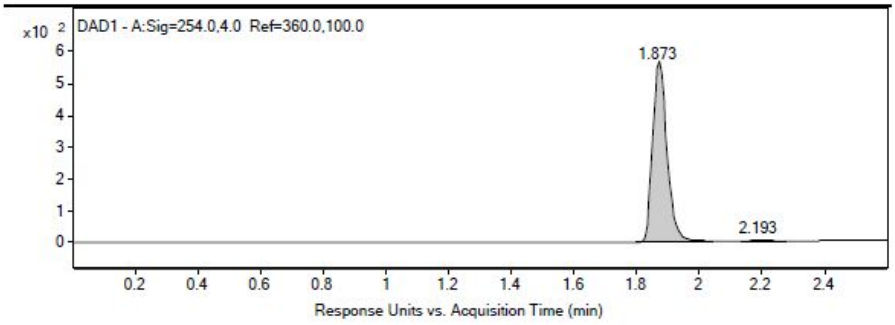
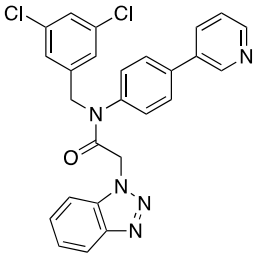

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 2
1.930
1.743
2.190
Response Units vs. Acquisition Time (min)



31


DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 2
1.873
2.193
Response Units vs. Acquisition Time (min)



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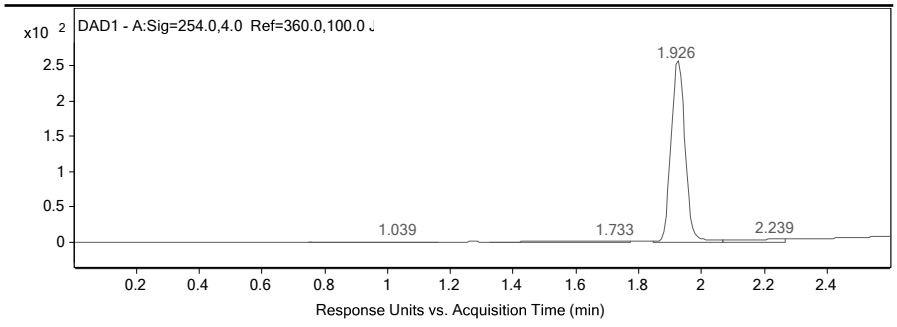
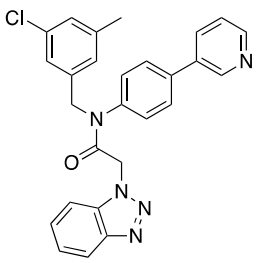

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 2
1.926
1.039
1.733
2.239
Response Units vs. Acquisition Time (min)



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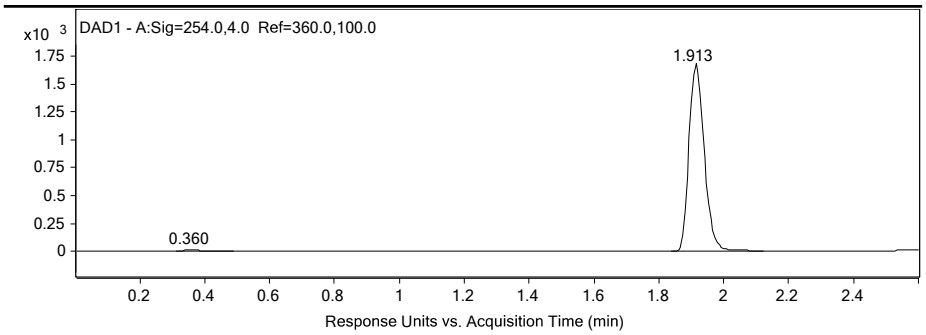
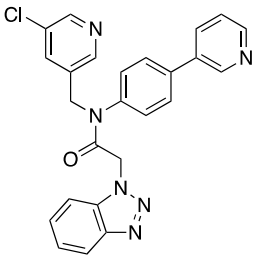

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 3
1.913
0.360
Response Units vs. Acquisition Time (min)



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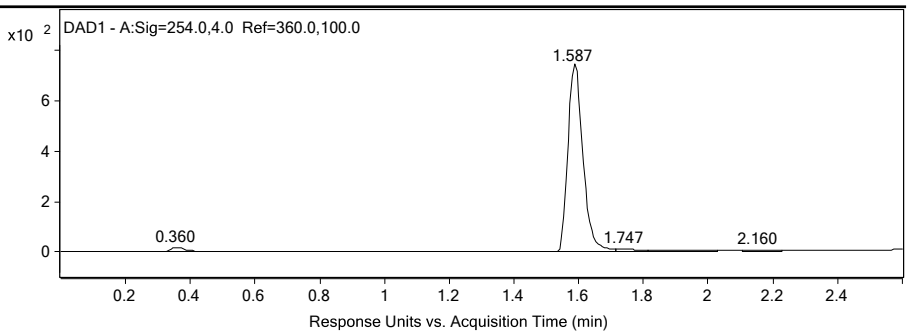
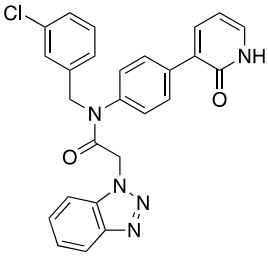
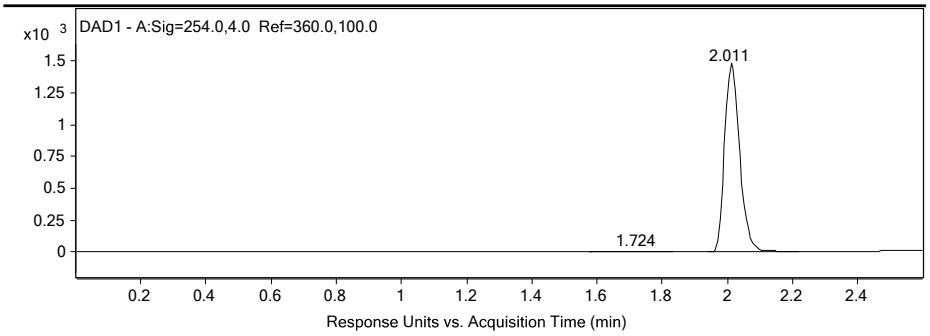
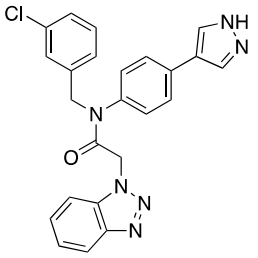

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 2
1.587
0.360
1.747
2.160
Response Units vs. Acquisition Time (min)



35


DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 3
2.011
1.724
Response Units vs. Acquisition Time (min)



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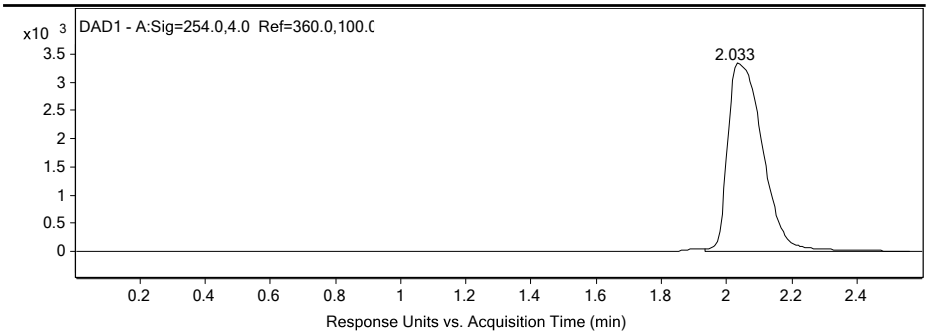
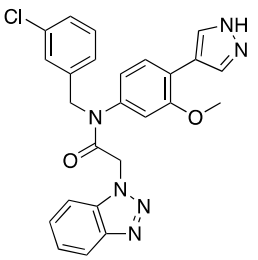

DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
x10 3
2.033
Response Units vs. Acquisition Time (min)



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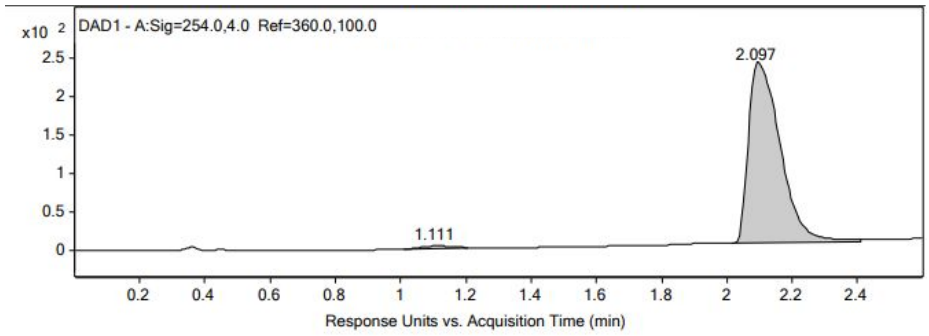
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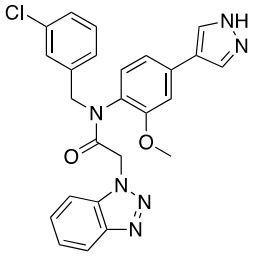
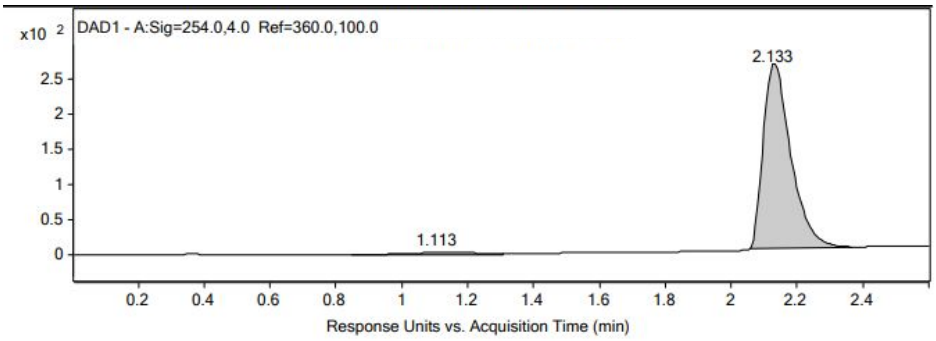
x10 2
2.097
1.111
Response Units vs. Acquisition Time (min)



38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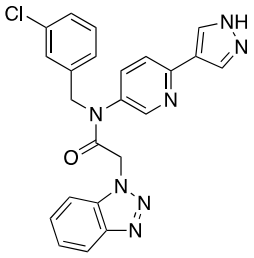
x10 2
2.133
1.113
Response Units vs. Acquisition Time (min)



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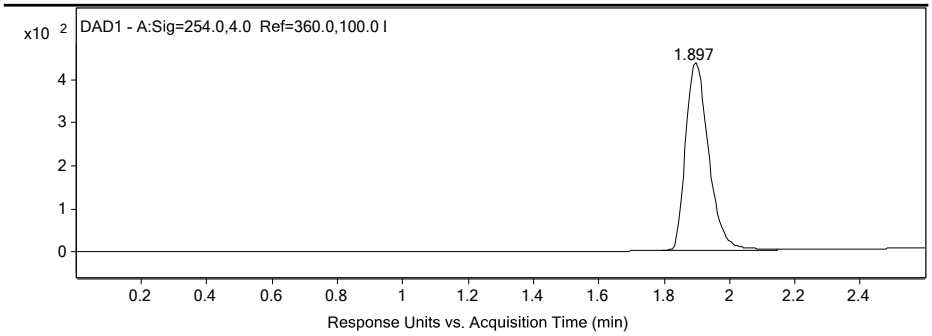
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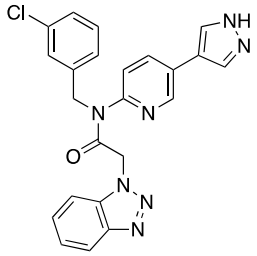
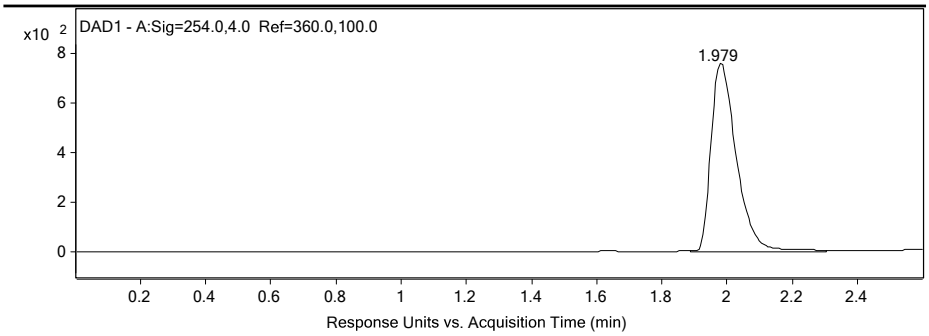
x10 2
1.897
Response Units vs. Acquisition Time (min)



40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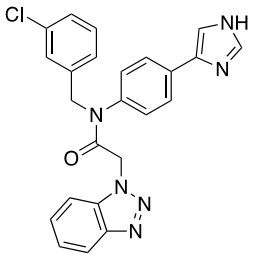
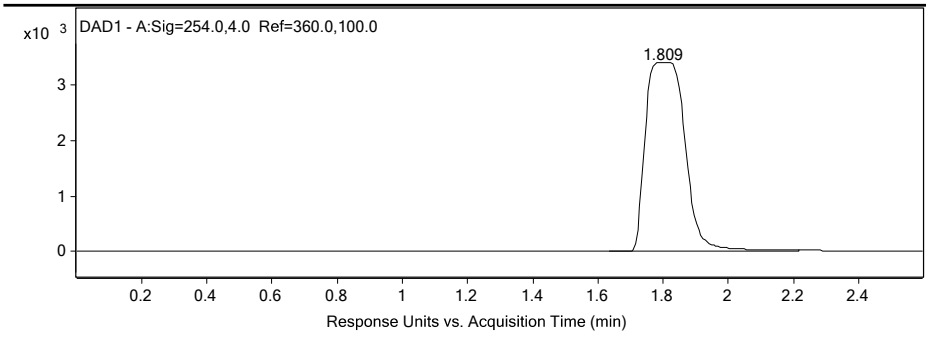
x10 2
1.979
Response Units vs. Acquisition Time (min)



41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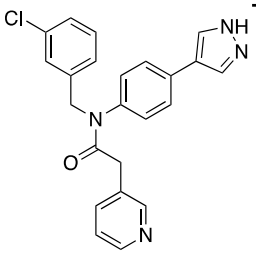
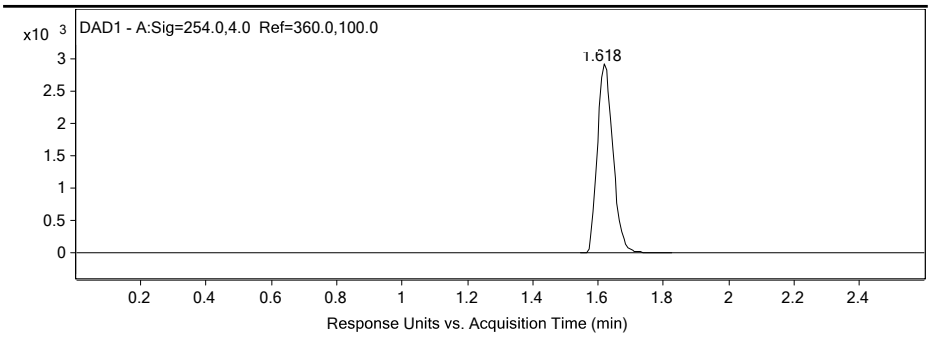
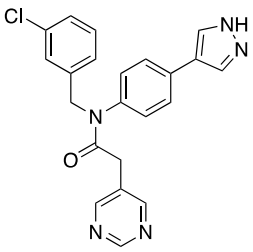
x10 3
1.809
Response Units vs. Acquisition Time (min)

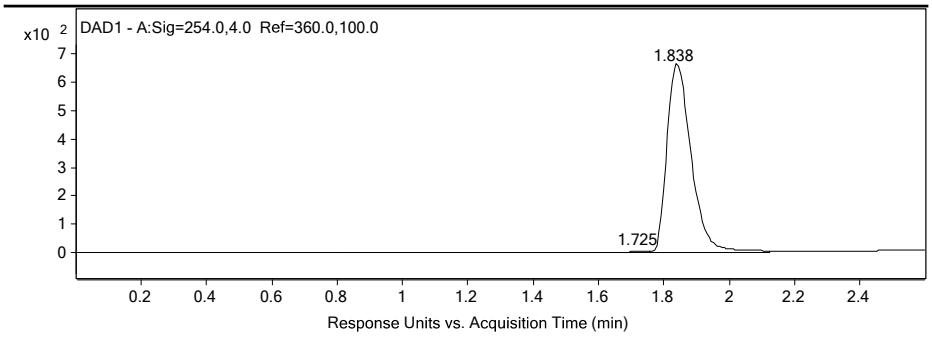

Cl
NH
N
N
O
N

42

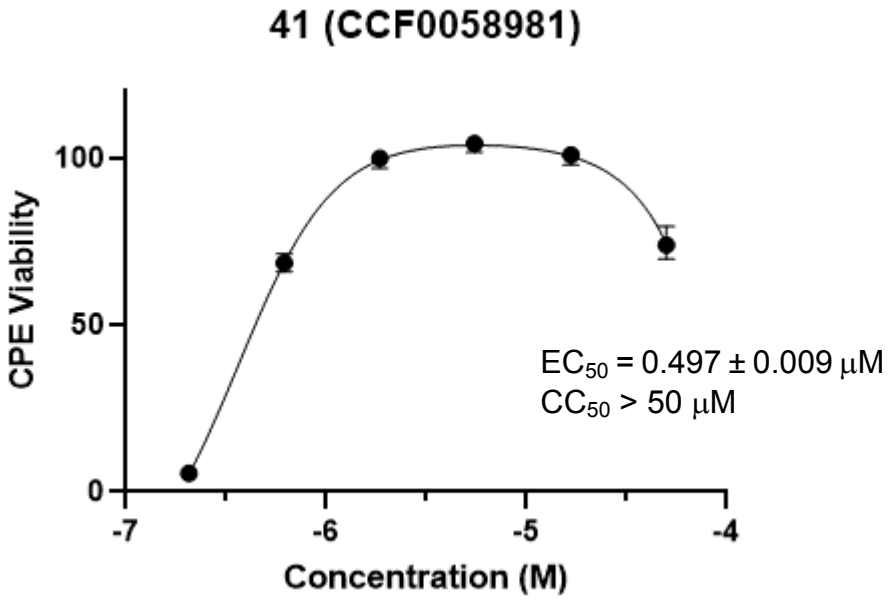

x10 3
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
1.618
3
2.5
2
1.5
1
0.5
0
0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4
Response Units vs. Acquisition Time (min)


Cl
NH
N
N
O
N
N

43


x10 2
DAD1 - A:Sig=254.0,4.0 Ref=360.0,100.0
1.838
1.725
7
6
5
4
3
2
1
0
0.2 0.4 0.6 0.8 1 1.2 1.4 1.6 1.8 2 2.2 2.4
Response Units vs. Acquisition Time (min)

Supplemental Figure S4. SARS-CoV-2 Vero E6 CPE Viability CRC **41**



Supplemental Table S5. Viability SARS-CoV-2 mock-infected Vero E6 cells using **41.**

[41] % Viability	**50 μM**	**16.6 μM**	**5.55 μM**	**1.85 μM**	**0.617 μM**	**0.206 μM**
	69.77	102.59	105.33	99.96	65.91	6.61
	79.50	97.90	104.39	101.21	71.29	3.49
	73.91	100.96	101.78	96.94	68.53	5.50