

Supporting Information

Discovery of highly potent fusion inhibitors with potential pan-coronavirus activity that effectively inhibit major COVID-19 variants of concern (VOCs)

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Keywords

Severe Acute Respiratory Syndrome (SARS), SARS-Cov, SARS-CoV-2, Middle East Respiratory Syndrome (MERS), MERS-CoV, COVID-19, pancoronavirus, fusion inhibitor

List of Items

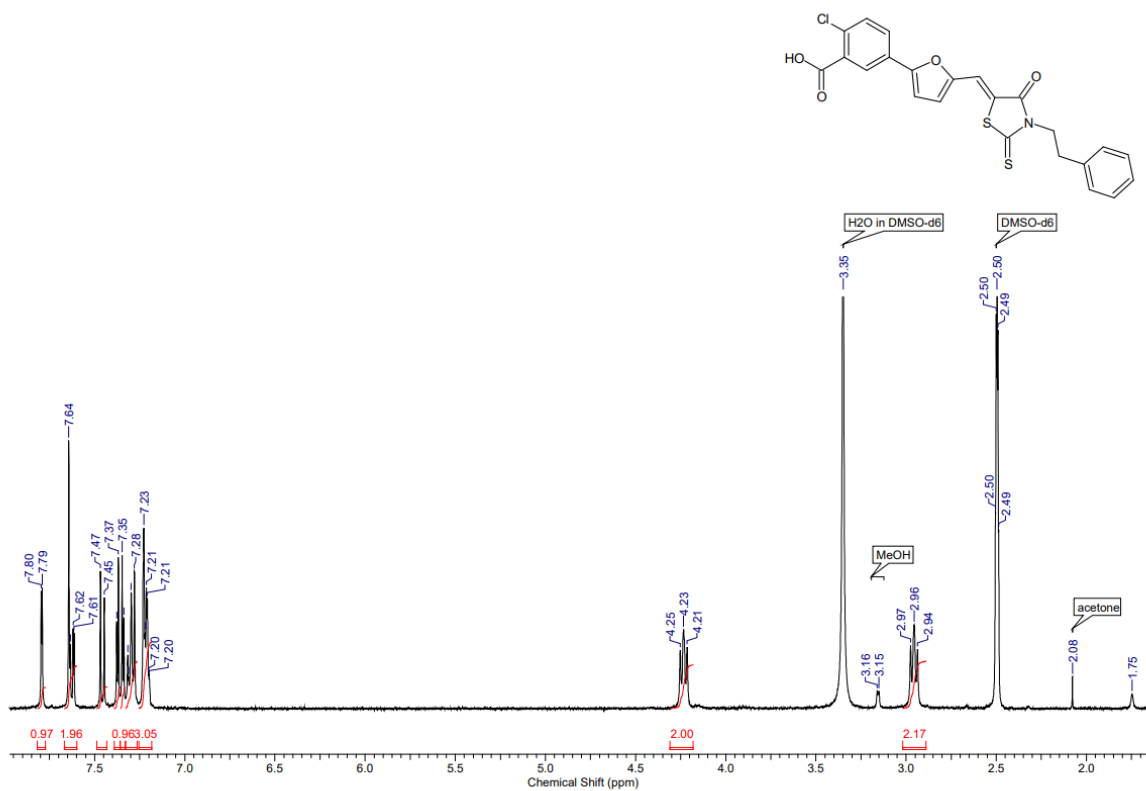
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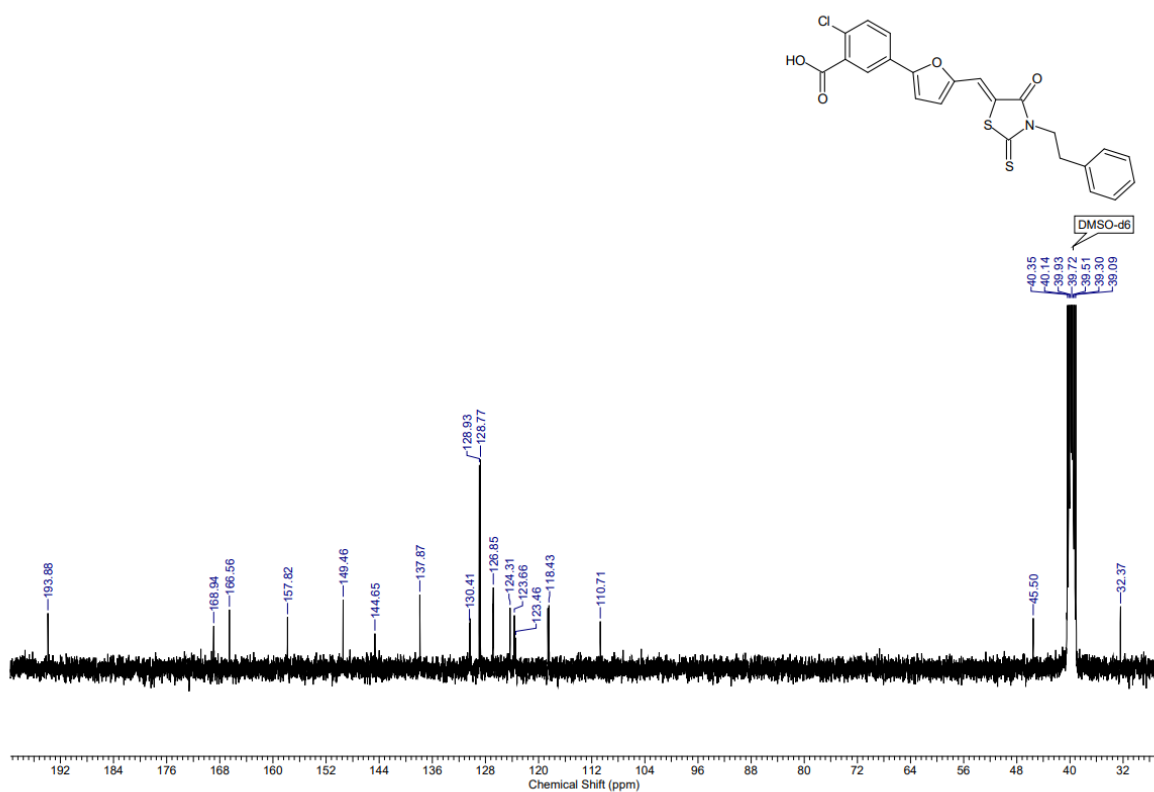
Experimental Section

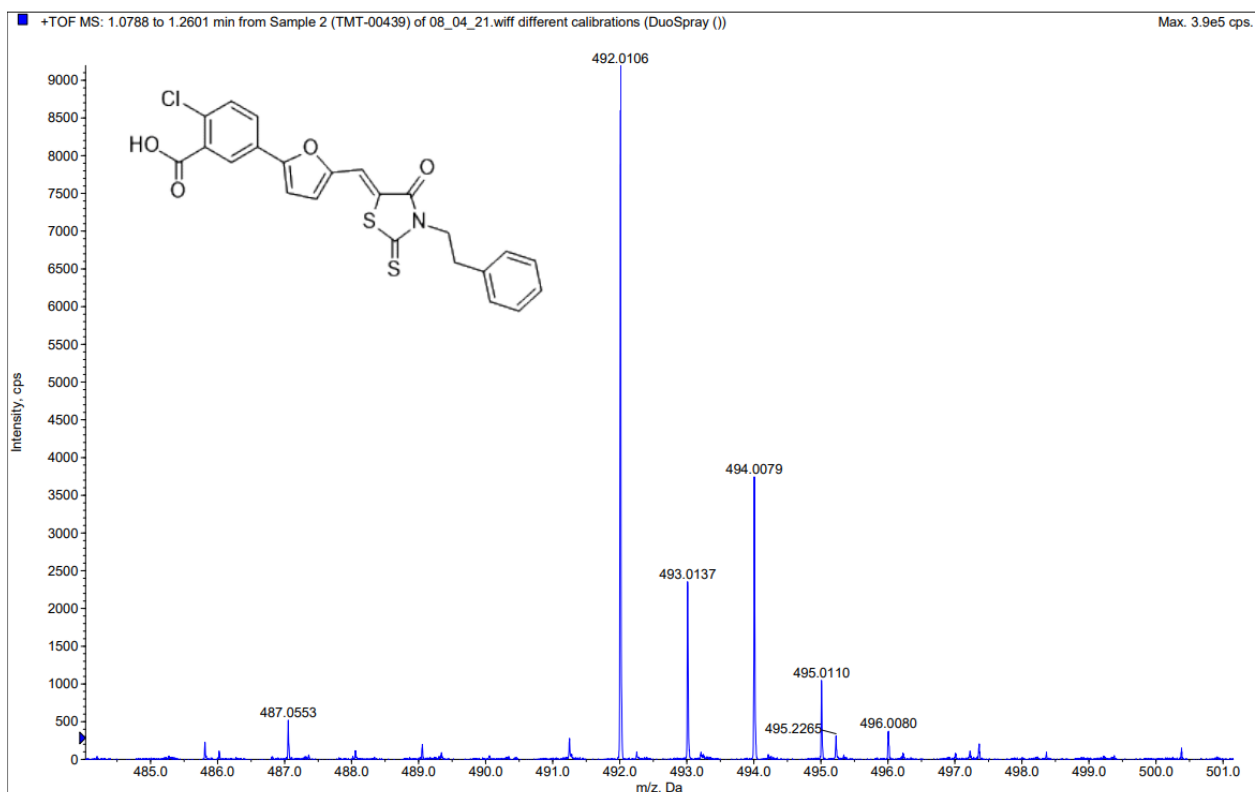
Purification and characterization of (Z)-2-Chloro-5-(5-((4-oxo-3-phenethyl-2-thioxothiazolidin-5-ylidene)methyl)furan-2-yl)benzoic acid (NBCoV1) purchased from Sigma-Aldrich.

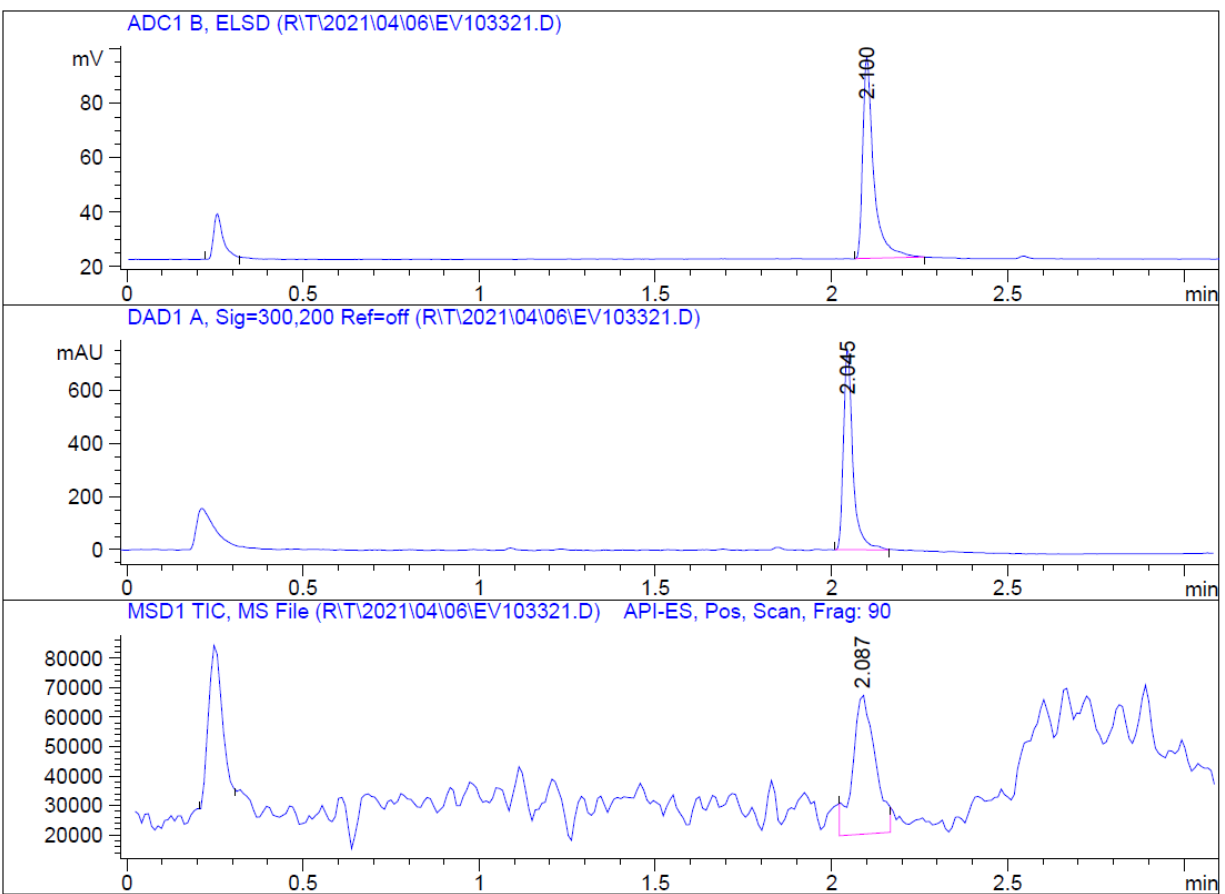
Common reagents and solvents were purchased from commercial suppliers and used without further purification unless otherwise stated. Tetrahydrofuran (THF) was distilled from sodium-benzophenone under an argon atmosphere. Reaction progress was monitored using analytical thin-layer chromatography (TLC) on pre-coated silica gel GF254 plates (Macherey Nagel GmbH & Co. KG, Düren, Germany), and spots were detected under UV light (254 and 366 nm). Compounds were purified with flash column chromatography with a silica gel and particle size of 40–63 μM (Merck, Darmstadt, Germany) as the stationary phase and hexane/ethyl acetate or dichloromethane/methanol mixtures as eluent systems. Nuclear magnetic resonance spectra were measured on a Bruker AV-400 NMR instrument (Bruker, Karlsruhe, Germany) in deuterated solvents (DMSO-d_6 , CDCl_3 , MeOD-d_4). Chemical shifts are expressed in ppm relative to DMSO-d_6 or MeOD-d_4 (2.50/3.31 for ^1H ; 39.52/49.00 for ^{13}C). The following abbreviations are used to set multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br. = broad. Measurements for verification and purity of the compounds were performed by LC/MS. LC–MS/MS data were obtained using a Dionex Ultimate 3000 liquid chromatograph (Dionex, USA) connected to an AB Sciex Qtrap 3200 mass spectrometer (AB Sciex, Canada). LC separation was carried out on a Shim-pack GIST C18-AQ (150 mm \times 2.1 mm, 3 μm , Shimadzu, Japan) column. Mobile phase consisted of the mixture of 0.1% (v/v) formic acid in water (A) and acetonitrile (B). Separation was performed in isocratic mode 10% : 90% (A : B). The mobile phase flow rate was 0.3 mL min^{-1} . The injection volume was 10 μL . Compounds were detected at $\lambda =$

254 nm. All high resolution mass spectra (HRMS) were measured on AB Sciex TripleTOF 5600+ instrument equipped with DuoSpray (ESI) ion source. Samples were directly injected in the ion source in acetonitrile or methanol solutions acidified by formic acid. The melting points were measured in open capillaries and presented without correction.



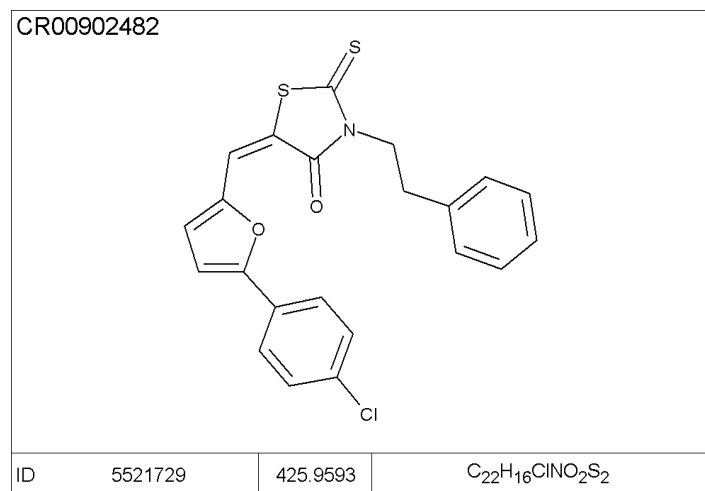






Analytical data on NBCoV15 purchased from Chembridge Corp (San Diego, CA)

Purity: 100%. MW: 425.9593 (Calc.); 425.9 (found).



Data File R:\HPLC\AUTO\CR009024\1BK-0901.D

Sample Name: CR009024P1-B-11

Instrument 1 15/04/2021 12:48:19 1

Column: ONYX MONOLITHIC, C18 50x3mm |1.8ml/min| Columns Reg Valve

Gradient: "A"->@2.0min->"B"(Hold 0.6min)->@0.2min->"A"->PostRun

PMP1, Solvent A : 0.1%TFA, 2.5%AcN in H₂O

PMP1, Solvent B : 0.1%TFA in AcN

PMP1, Solvent C : --NOT USED--

PMP1, Solvent D : --NOT USED--

Ionization mode : API-ES Positive

Signal 1: ADC1 B, ELSD

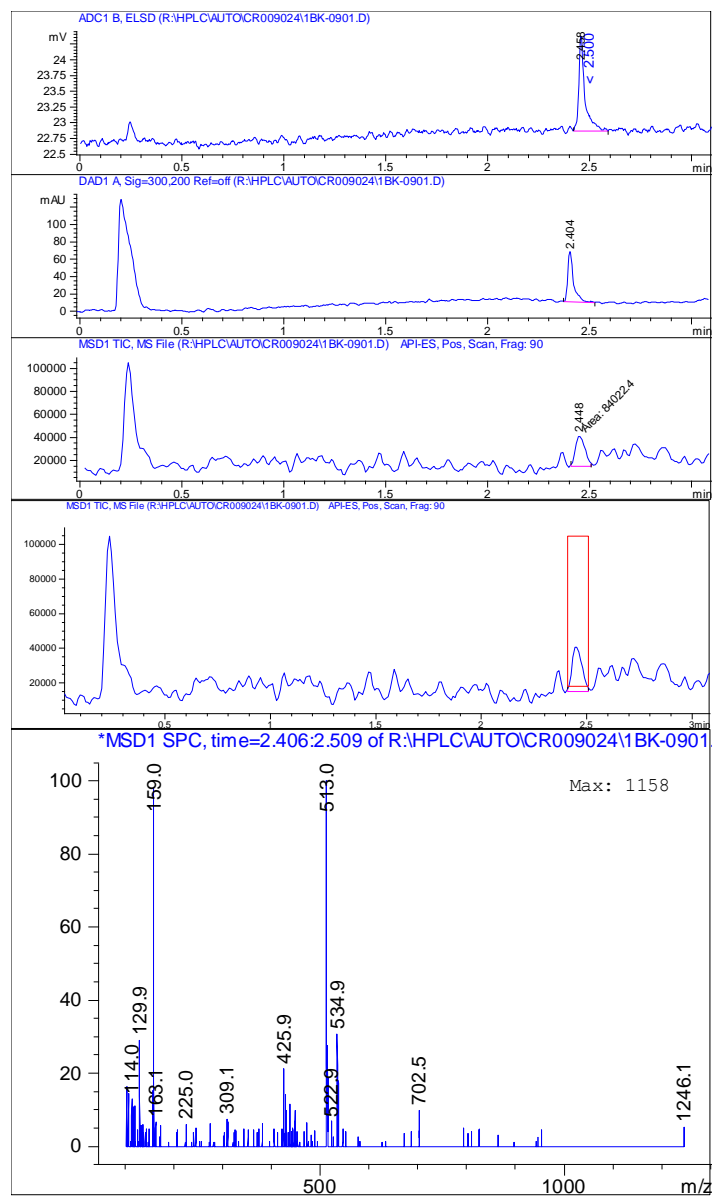
Peak #	RetTime [min]	Type	Width [mV*s]	Area [mV]	Height %	Area
1	2.458	PPA	0.0312	3.15085	1.51854	100.0000
Totals :			3.15085	1.51854		

Signal 2: DAD1 A, Sig=300,200 Ref=off

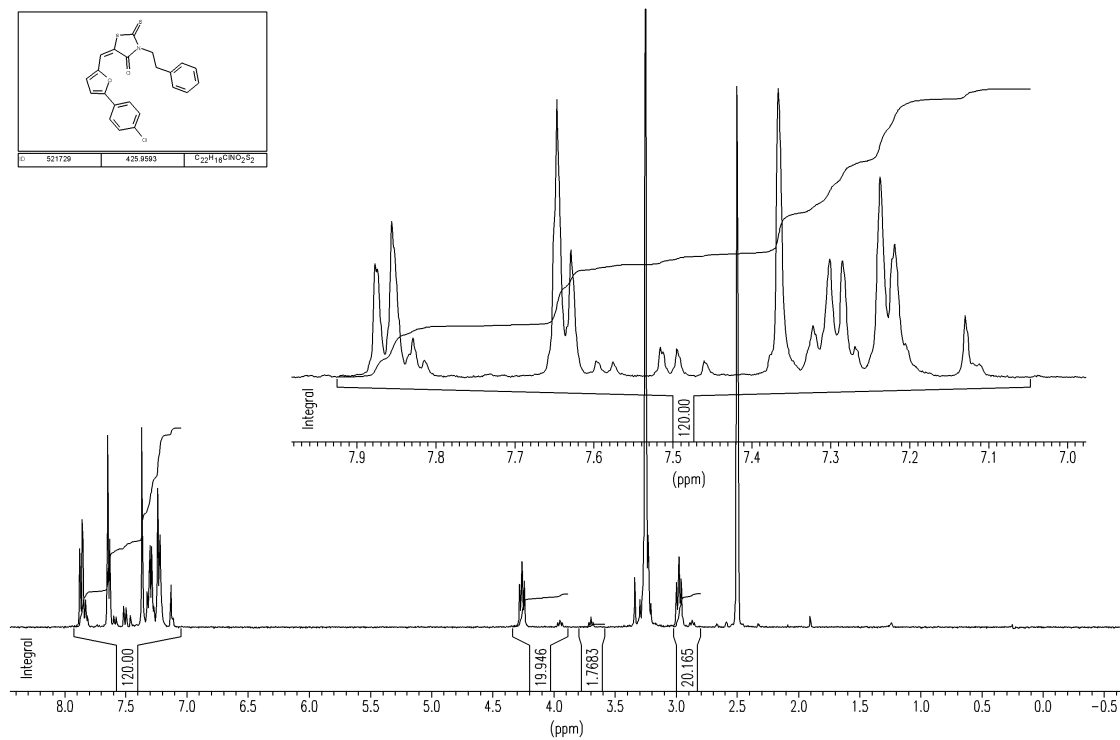
Peak #	RetTime [min]	Type	Width [mAU*s]	Area [mAU]	Height %	Area
1	2.404	BP	0.0299	113.99640	58.00123	100.0000
Totals :			113.99640	58.00123		

Signal 3: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height %	Area
1	2.448	MM	0.0526	8.40224e4	2.66246e4	100.0000
Totals :				8.40224e4	2.66246e4	

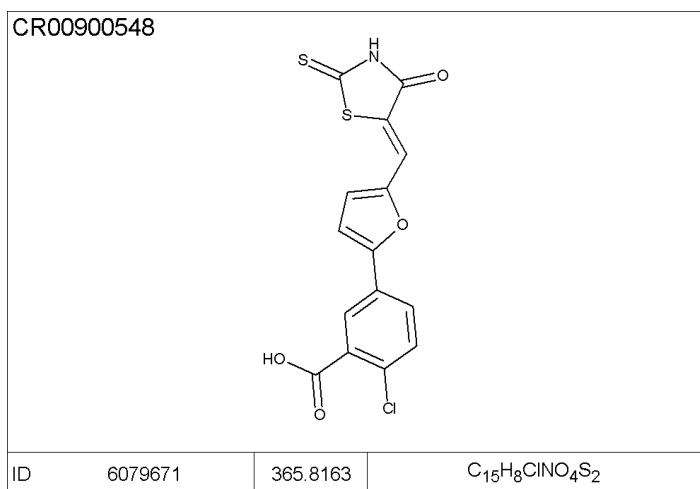
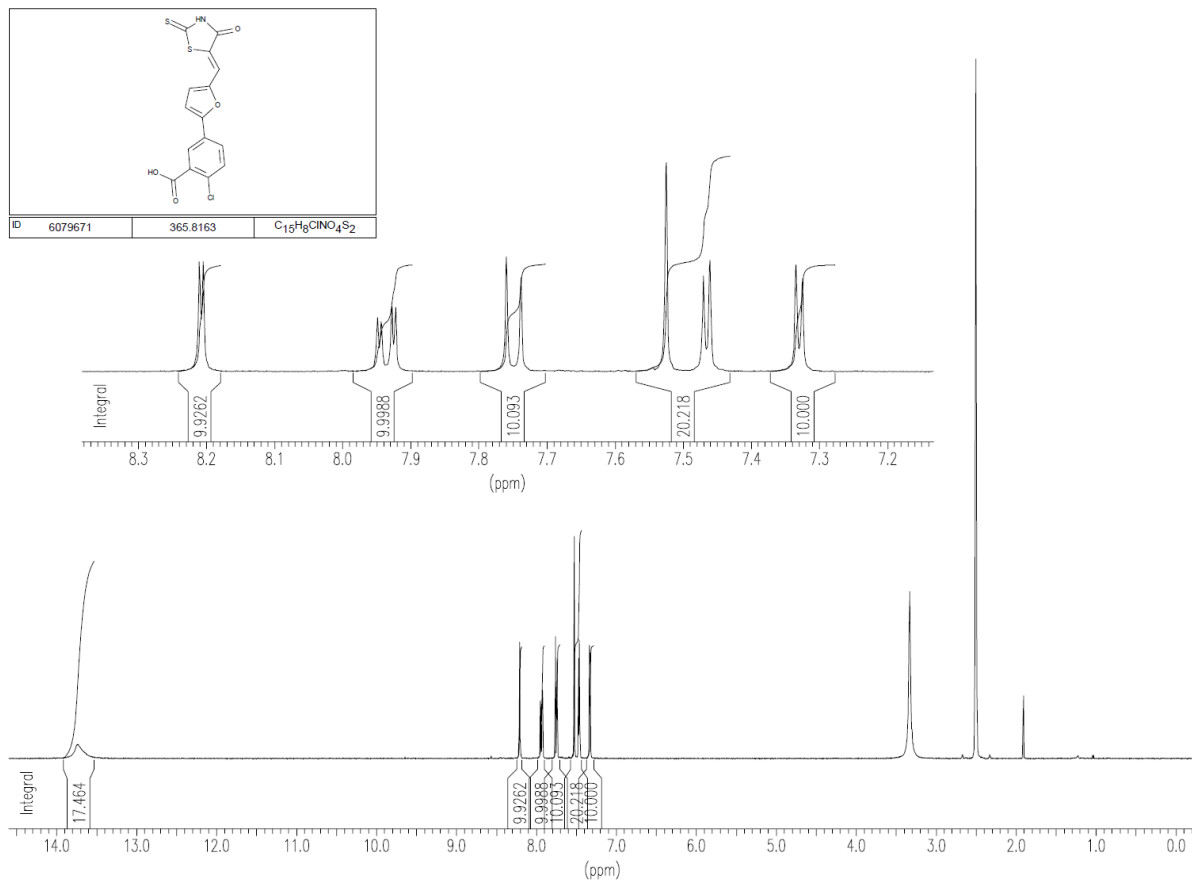


521729x2 in DMSO.



Analytical data on NBCoV17 purchased from Chembridge Corp (San Diego, CA)

6079671X2 DMSO-D6 Dsh 150723-2/09



Data File D:\DATA\MICRA\2HF-0101.D

Sample Name: CR009005P2-H-06
 Instrument 1 23/07/2015 16:34:24 6
 Column: Onyx Monolithic C18 50x4.6mm | 3.75ml/min | Columns Reg Valve
 Gradient: "C"->@2.2min->"D"(Hold 0.4min)->@0.2min->"C"->PostRun
 PMP1, Solvent A : 0.1%TFA in Acn/H2O (2.5:97.5)
 PMP1, Solvent B : 0.1%TFA in AcN
 PMP1, Solvent C : 0.1%FA in Acn/H2O (2.5:97.5)
 PMP1, Solvent D : 0.1%FA in AcN
 Ionization mode : APCI Negative

Signal 1: ADC1 B, ELSD

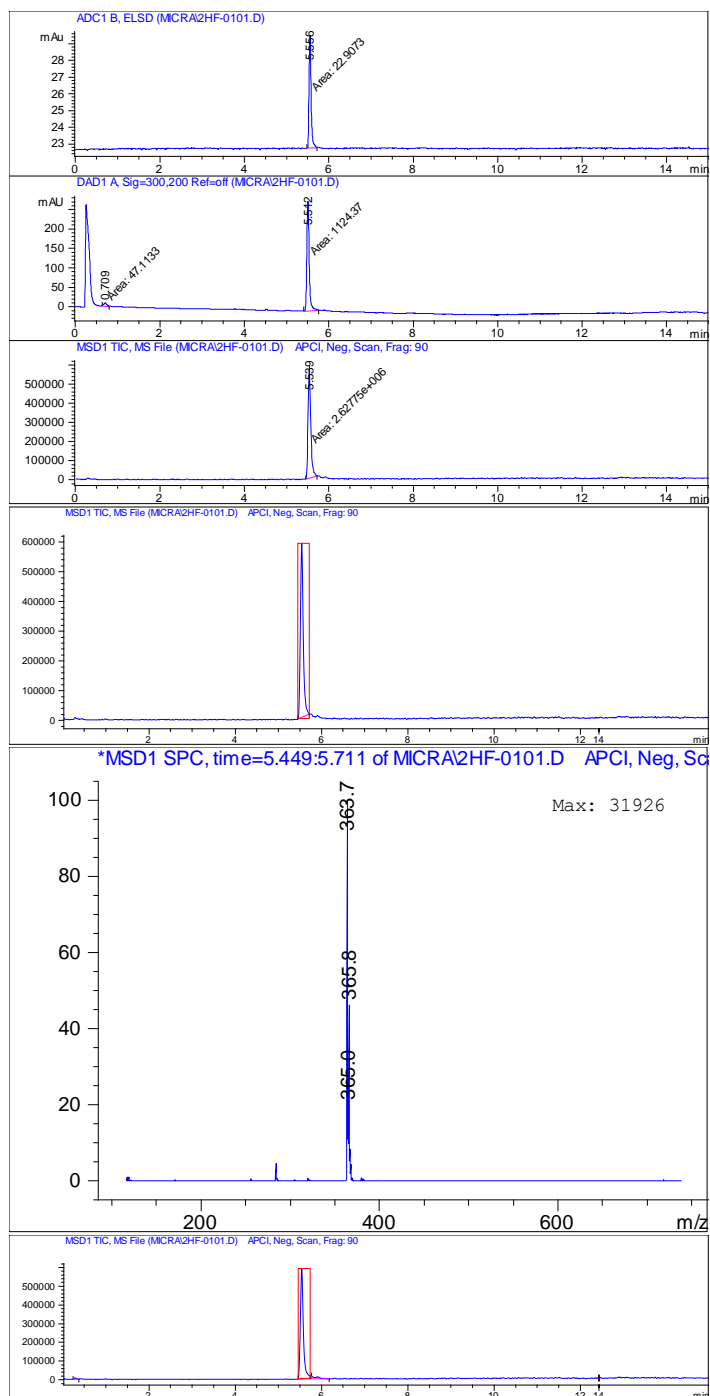
Peak #	RetTime [min]	Type	Width [mAu*s]	Area [mAu]	Height %	Area
1	5.556	MM	0.0550	22.90727	6.93644	100.0000
Totals :			22.90727	6.93644		

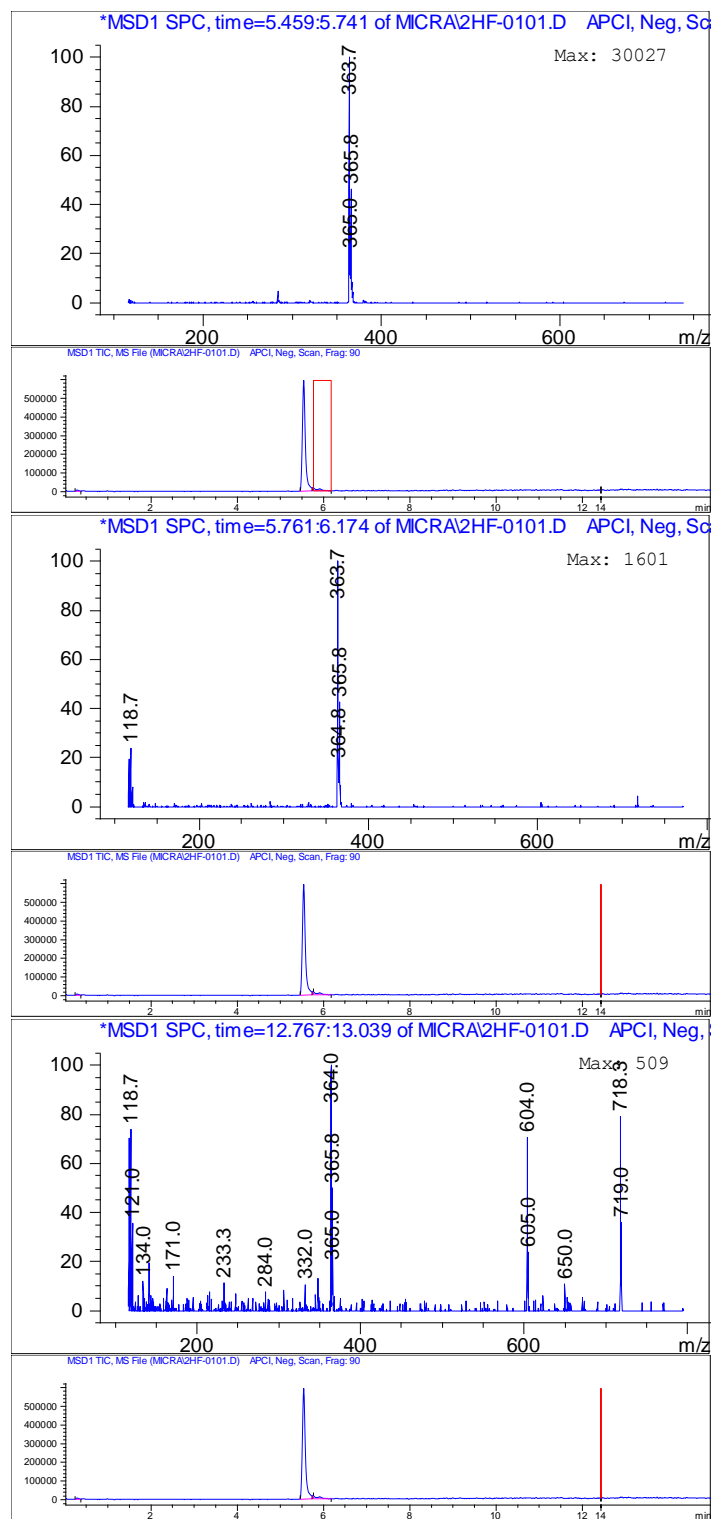
Signal 2: DAD1 A, Sig=300,200 Ref=off

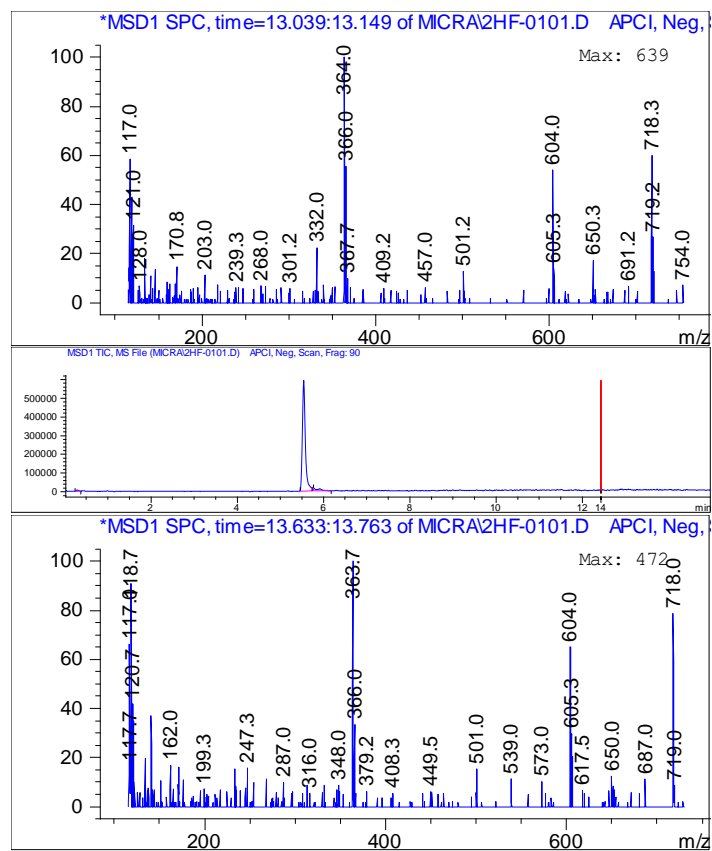
Peak #	RetTime [min]	Type	Width [mAU*s]	Area [mAU]	Height %	Area
1	0.709	MM	0.0889	47.11327	8.83276	4.0217
2	5.512	MM	0.0626	1124.37195	299.48669	95.9783
Totals :			1171.48522	308.31946		


Signal 3: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width	Area	Height %	Area
1	5.539	MM	0.0744	2.62775e6	5.89026e5	100.0000
Totals :			2.62775e6	5.89026e5		







			<p>ID 6699319</p> <p>387.8011</p> <p>$C_{18}H_{10}ClNO_5S$</p>
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S19

Sample Name: CR009024P1-C-12
Instrument 1 20/04/2021 14:50:59 1
Column: Onyx C18 50x4.6mm | 3.75ml/min | Columns Reg Valve
Gradient: "A"->@2.2min->"B"(Hold 0.4min)->@0.2min->"A"->PostRun
PMP1, Solvent A : 0.1TFA in 2.5%AcN/W
PMP1, Solvent B : 0.1TFA in AcN
PMP1, Solvent C : 0.1FA in 2.5%AcN/W
PMP1, Solvent D : 0.1FA in AcN
Ionization mode : API-ES Negative

Signal 1: ADC1 A, ADC1 ELSD

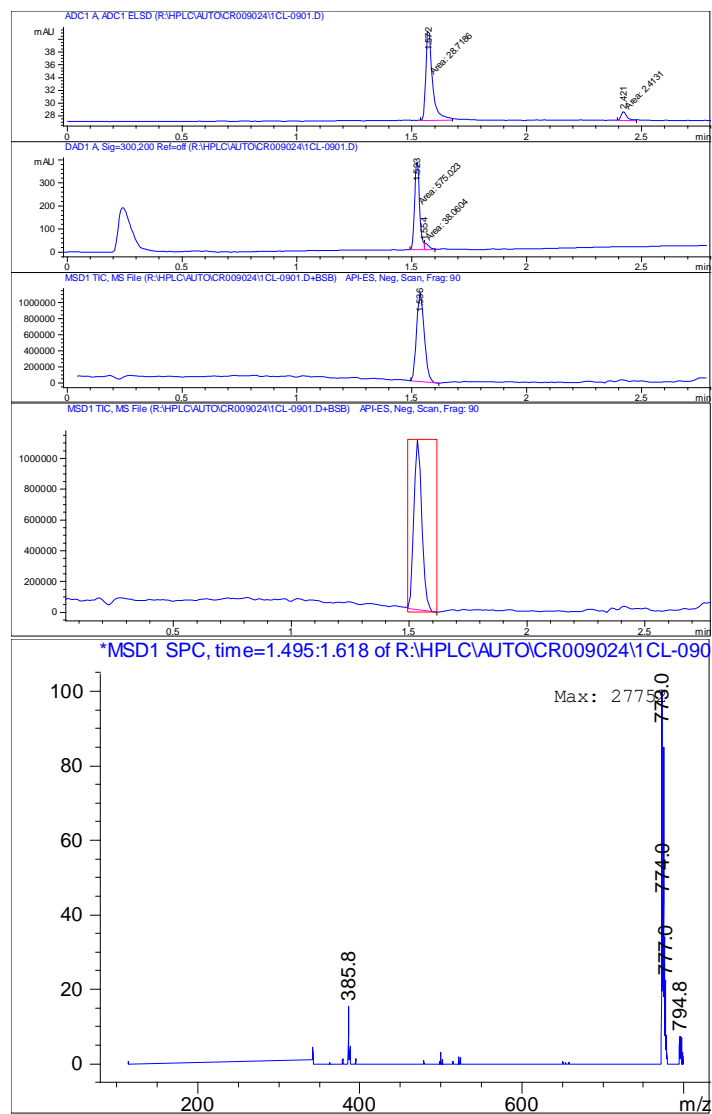
Peak #	RetTime [min]	Type	Width [mAU*s]	Area [mAU]	Height	Area %
1	1.572	MM	0.0317	28.71860	15.10031	92.2487
2	2.421	MM	0.0276	2.41310	1.45476	7.7513
Totals :				31.13171	16.55507	

Signal 2: DAD1 A, Sig=300,200 Ref=off

Peak #	RetTime [min]	Type	Width [mAU*s]	Area [mAU]	Height	Area %
1	1.523	MF	0.0248	575.02313	386.74631	93.7920
2	1.554	FM	0.0211	38.06044	30.04487	6.2080
Totals :				613.08357	416.79118	

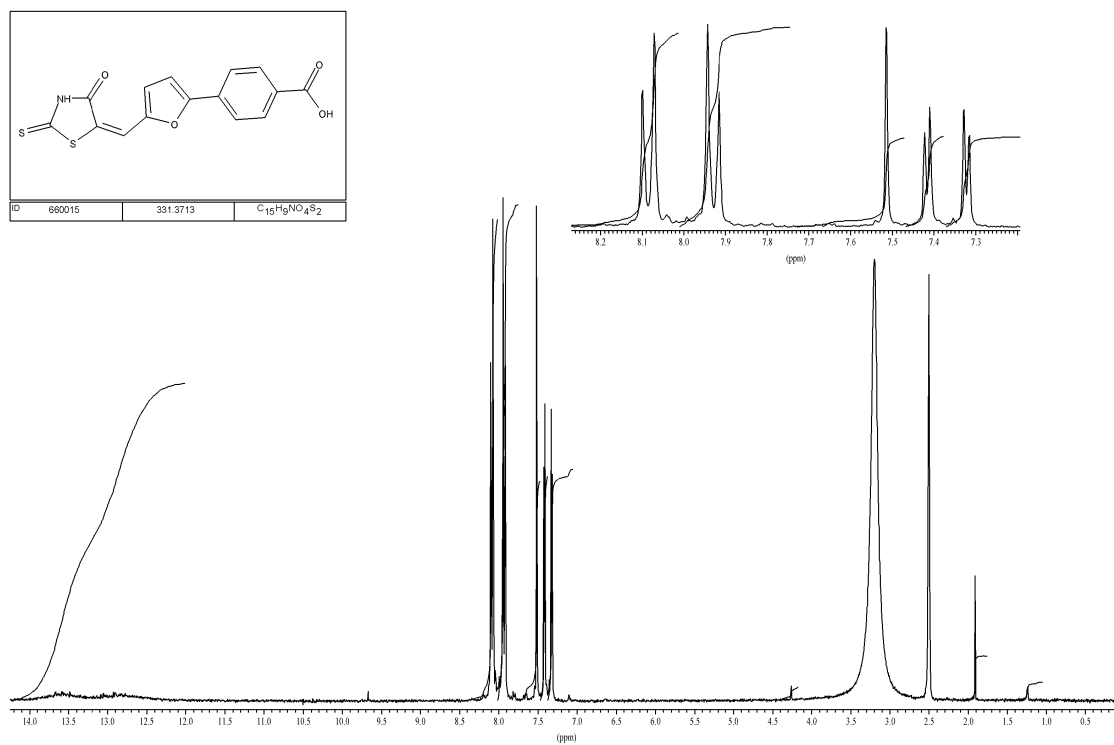
Signal 3: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width	Area	Height	Area %
1	1.536	BP	0.0365	2.58954e6	1.10936e6	100.0000
Totals :				2.58954e6	1.10936e6	

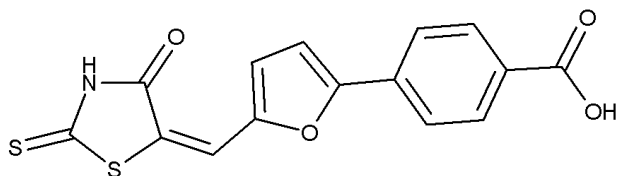


Analytical data on NBCoV34 purchased from Chembridge Corp (San Diego, CA)

660015-A



CR00902485



ID	5660015	331.3713	C ₁₅ H ₉ NO ₄ S ₂
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Data File D:\DATA\MICRA\1EK-0301.D

Sample Name: CR009024P1-E-11

Instrument 1 20/04/2021 16:54:25 1

Column: Onyx C18 50x4.6mm | 3.75ml/min | Columns Reg Valve

Gradient: "A"->@2.0min->"B"(Hold 0.4min)->@0.2min->"A"->PostRun

PMP1, Solvent A : 0.1TFA in 2.5%AcN/W

PMP1, Solvent B : 0.1TFA in AcN

PMP1, Solvent C : 0.1FA in 2.5%AcN/W

PMP1, Solvent D : 0.1FA in AcN

Ionization mode : API-ES Negative

Signal 1: ADC1 A, ADC1 ELSD

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.463	MM	0.0391	1.73849	7.40851e-1	91.3606
2	2.409	MM	0.0206	1.64398e-1	1.32726e-1	8.6394
Totals :				1.90288	8.73577e-1	

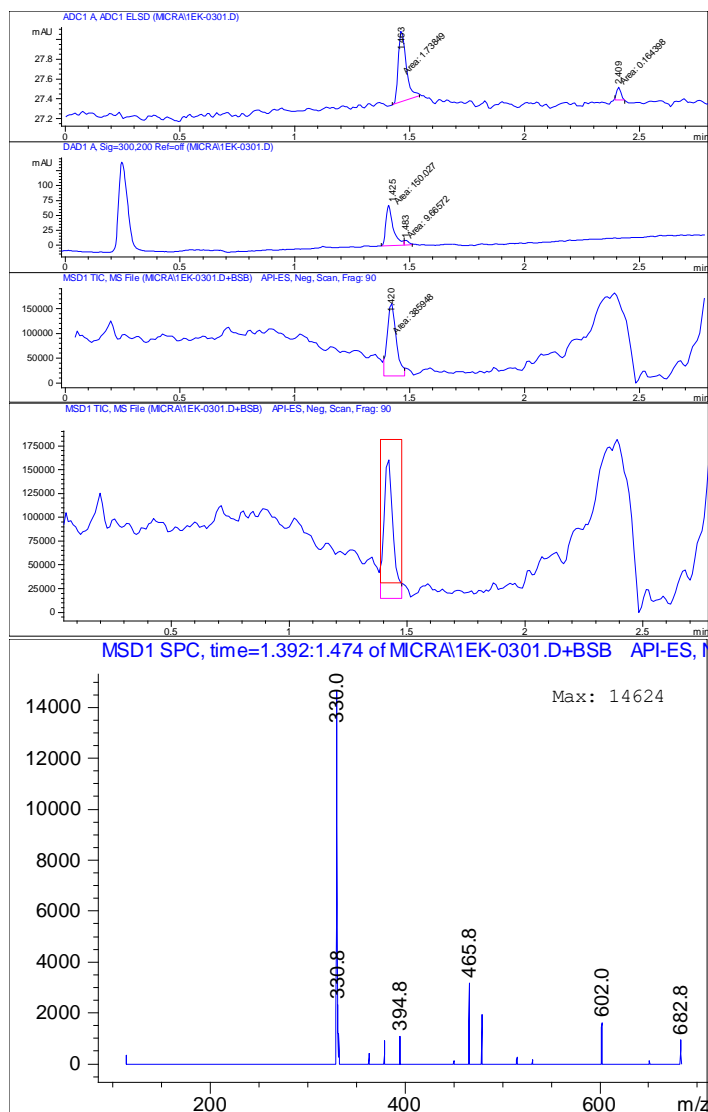
Signal 2: DAD1 A, Sig=300,200 Ref=off

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	1.425	MF	0.0366	150.02690	68.30225	93.9473
2	1.483	FM	0.0217	9.66572	7.43823	6.0527
Totals :				159.69263	75.74048	

Signal 3: MSD1 TIC, MS File

Peak #	RetTime [min]	Type	Width [min]	Area	Height	Area %
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	1	1.420 MM	0.0429	3.85948e5	1.49882e5	100.0000
Totals :				3.85948e5	1.49882e5	



In vitro ADME Study

1. EQUILIBRIUM SOLUBILITY

1.1. Experimental Procedure

The equilibrium solubility of one test article was measured in pH 7.4 aqueous buffer. The buffer was prepared by combining 50 mL of 0.2 M KH₂PO₄ with 150 mL of H₂O, and then adjusting to pH 7.4 with 10 N NaOH. At least 1 mg of powder for each test article was combined with 1 mL of buffer to make a ≥ 1 mg/mL mixture. These samples were shaken on a Thermomixer® overnight at room temperature. The samples were then passed through a 0.45 μ m PTFE syringe filter. The filtrate was sampled and diluted in duplicate 10-, 100-, 1000-, and 10000-fold into a mixture of 1:1 buffer:acetonitrile (ACN) prior to analysis. All samples were assayed by LC-MS/MS using electrospray ionization against standards prepared in a mixture of 1:1 assay buffer:ACN. Standard concentrations ranged from 1.0 μ M to 0.3 nM. Analytical conditions are outlined in Appendix 1.

1.2. Experimental Results

Test Article	Solubility in Phosphate Buffer (μ M)		
	R1	R2	AVG
NBCoV1	4.35	5.08	4.72

2. P-GP SUBSTRATE ASSESSMENT

2.1. Experimental Procedure

Caco-2 cells (clone C2BBE1) were obtained from American Type Culture Collection (Manassas, VA). Cell monolayers were grown to confluence on collagen-coated, microporous membranes in 12-well assay plates. Details of the plates and their certification are shown below. The permeability assay buffer was Hanks' balanced salt solution (HBSS) containing 10 mM HEPES and 15 mM glucose at a pH of 7.4. The buffer in the receiver chamber also contained 1% bovine serum albumin. The dosing solution concentration was 5 μ M of test article in the assay buffer +/- 1 μ M valsopodar. Cells were first pre-incubated for 30 minutes with HBSS containing +/- 1 μ M valsopodar. Cell monolayers were dosed on the apical side (A-to-B) or basolateral side (B-to-A) and incubated at 37°C with 5% CO₂ in a humidified incubator. Samples were taken from the donor and receiver chambers at 120 minutes. Each determination was performed in duplicate. The flux of lucifer yellow was also measured post-experimentally for each monolayer to ensure no damage was inflicted to the cell monolayers during the flux period. All samples were assayed by LC-MS/MS using electrospray ionization. Analytical conditions are outlined in Appendix 1. The apparent permeability (P_{app}) and percent recovery were calculated as follows:

$$P_{app} = (dC_r / dt) \times V_r / (A \times C_A) \quad (1)$$

$$\text{Percent Recovery} = 100 \times ((V_r \times C^{final}) + (V_d \times C_{dfinal})) / (V_d \times C_N) \quad (2)$$

Where,

dC_r / dt is the slope of the cumulative receiver concentration versus time in μ M s⁻¹;
 V_r is the volume of the receiver compartment in cm³;
 V_d is the volume of the donor compartment in

c
m
3
;
A
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S
t

he area of the insert (1.13 cm² for 12-well);
 CA is the average of the nominal dosing concentration and the measured 120
 minutedonor concentration in μM;
 CN is the nominal concentration of the dosing solution in μM;
 C^{final} is the cumulative receiver concentration in μM at the end of the incubation period;
 Cd^{final} is the concentration of the donor in μM at the end of the incubation period.

Efflux ratio (ER) is defined as P_{app} (B-to-A) / P_{app} (A-to-B).

2.2. Cell Batch Quality Control Results

Plates	12-well	
Seed Date	20Jan2021	
Passage Number	67	
Age at QC (days)	20	
Age at Experiment (days)	27	Acceptance Criteria
Atenolol Papp, 10 ⁻⁶ cm/s	0.193	≤ 0.5
Propranolol Papp, 10 ⁻⁶ cm/s	19.7	10-30
Digoxin A-to-B Papp, 10 ⁻⁶ cm/s	0.340	N/A
Digoxin B-to-A Papp, 10 ⁻⁶ cm/s	12.9	N/A
Digoxin Efflux Ratio	37.9	≥ 10

2.3. Experimental results

Test Article	Direction	Recovery (%)	Papp (10 ⁻⁶ cm/s)			Efflux Ratio	P-gp Substrate Classification
			R1	R2	AVG		
NBCoV1	A-to-B	46.2	18.1	15.6	16.9	1.21	Negative
	B-to-A	54.2	17.8	23.1	20.4		
NBCoV1	A-to-B	34.4	20.4	20.6	20.5	1.15	
+ 1 µM Valspodar	B-to-A	56.6	20.9	26.4	23.6		

P-gp Substrate Classification:

CER ≥ 1.0 without valsopodar, and reduced by ≥ 50% with valsopodar: **Positive**

CER ≥ 1.0 without valsopodar, and reduced by < 50% with valsopodar: **Negative**

CER < 1.0 without and with valsopodar: **Negative**

CER = Corrected Efflux Ratio = ER – 1

3. PLASMA PROTEIN BINDING

3.1. Experimental Procedure

Studies were carried out in mixed-gender human plasma (Lot# AS1650-36), obtained from BioIVT and collected on K2EDTA. A Pierce Rapid Equilibrium Dialysis (RED) device was used for all experiments. Stock solutions of the test article and control compound were first prepared in DMSO. Aliquots of the DMSO solutions were dosed into 1.0 mL of plasma at a dosing concentration of 5 µM for the test article and 10 µM for the co-dosed control compound, warfarin. Plasma (300 µL), containing test article and control compound, was loaded into two wells of the 96-well dialysis plate. Blank phosphate-buffered saline (PBS) (500 µL) was added to each corresponding receiver chamber. The device was then placed into an enclosed heated rocker that was pre-warmed to 37°C, and allowed to incubate for four hours. After 4 hours of incubation, both sides were sampled.

Aliquots (50 µL for donor, 200 µL for receiver) were removed from the chambers and placed into a 96-well plate. Plasma (50 µL) was added to the wells containing the receiver samples, and 200 µL of PBS was added to the wells containing the donor samples. Two volumes of acetonitrile (ACN) were added to each well, and the plate was mixed and then centrifuged at 3,000 rpm for 10 minutes. Aliquots of the supernatant were removed, diluted 1:1 into water, and analyzed by LC-MS/MS.

Protein binding values were calculated as follows:

$$\% \text{ Bound} = [(PARR \text{ in Donor} - PARR \text{ in Receiver}) / (PARR \text{ in Donor})] \times 100$$

PARR = peak area response ratio to internal standard, including applicable dilution factors.

3.2. Experimental Results

Test Article	% Bound					
	Test Article			Warfarin		
	R1	R2	AVG	R1	R2	AVG
NBCoV1	> 99.5	> 99.5	> 99.5	98.4	98.2	98.3

Warfarin binding acceptance criteria: ≥ 98.0 % bound

4. STABILITY IN LIVER MICROSOMES

4.1. Experimental Procedure

Mixed-gender human liver microsomes (Lot# 1010420) were purchased from XenoTech. The reaction mixture, minus NADPH, was prepared as described below. The test article was added into the reaction mixture at a final concentration of 1 μ M. The control compound, testosterone, was run simultaneously with the test article in a separate reaction. The reaction mixture (without cofactor) was equilibrated in a shaking water bath at 37°C for 5 minutes. The reaction was initiated by the addition of the cofactor, and the mixture was incubated in a shaking water bath at 37°C. Aliquots (100 μ L) were withdrawn at 0, 15, 30, 60, 90, and 120 minutes. Test article and testosterone samples were immediately combined with 400 μ L of ice-cold 50/50 acetonitrile (ACN)/H₂O containing 0.1% formic acid and internal standard to terminate the reaction. The samples were then mixed and centrifuged to precipitate proteins. All samples were assayed by LC-MS/MS using electrospray ionization. Analytical conditions are outlined in Appendix 1. The peak area response ratio (PARR) to internal standard was compared to the PARR at time 0 to determine the percent remaining at each time point. Half-lives and clearance were calculated using GraphPad software, fitting to a single-phase exponential decay equation.

4.2. Reaction Composition

Liver Microsomes 0.5 mg/mL

NADPH (cofactor) 1 mM Potassium Phosphate, pH 7.4 100 mM Magnesium Chloride 5 mM

Test Article 1 μ M

4.3. Experimental Results

Test Article	% Remaining of Initial (n=1)						Half-life (min)	CLint (mL/min/mg protein)
	0 min	15 min	30 min	60 min	90 min	120 min		
NBCoV1	100	101	80.5	68.9	69.5	41.6	112	0.0124

Control Compound	Species	Half-life (min)	CLint (ml/min/mg protein)	Acceptable Range (t1/2, min)
Testosterone	Human	11.3	0.122	≤ 41

5. CYP IC50

5.1. Experimental Procedure

The test articles, at eight concentrations (0-10 μ M), were incubated with pooled HLM (0.25 mg protein/mL) in phosphate buffer (100 mM, pH 7.4) containing MgCl₂ (5 mM), NADPH (1 mM), and an individual CYP probe substrate (at approximately K_m). The reaction mixture minus NADPH was equilibrated in a shaking water bath at 37°C for 5 minutes. The reaction was

initiated by the addition of NADPH, followed by incubation at 37°C for 10-30 minutes depending on the individual CYP isoform. The reaction was terminated by the addition of two volumes of ice-cold acetonitrile. Negative (vehicle) controls were conducted without the test article. Positive controls were performed in parallel at a single concentration using known CYP inhibitors. After the removal of protein by centrifugation at 1640g (3000 rpm) for 10 minutes at 4°C, the supernatants were transferred to a 96-well plate and diluted with water containing internal standard (stable isotope-labeled CYP probe metabolite). The formation of CYP probe metabolite was determined by LC-MS/MS.

5.2. CYP Probe Substrates and Metabolites

CYP	Probe Substrate	Metabolite	Incubation Time (min)	Positive Control Inhibitor
CYP1A2	Phenacetin (63 µM)	Acetaminophen	20	α-Naphthoflavone (1 µM)
CYP2B6	Bupropion (75 µM)	OH bupropion	20	Thio-TEPA (30 µM)
CYP2C8	Amodiaquine (2 µM)	Desethylamodiaquine	20	Montelukast (5 µM)
CYP2C9	Diclofenac (10 µM)	4'-OH diclofenac	20	Sulfaphenazole (10 µM)
CYP2C19	S-mephenytoin (40 µM)	4'-OH mephenytoin	30	(+)-N-3-benzylrivanol (5 µM)
CYP2D6	Bufuralol (7 µM)	1'-OH bufuralol	20	Quinidine (1 µM)
CYP3A	Midazolam (2.5 µM)	1'-OH midazolam	10	Ketoconazole (1 µM)
	Testosterone (55 µM)	6β-OH testosterone	10	Ketoconazole (1 µM)

5.3. Experimental Results

CYP	% of Control Enzyme Activity (n=1) ^a								IC ₅₀ (µM)
	0 µM	0.0137 µM	0.0412 µM	0.123 µM	0.370 µM	1.11 µM	3.33 µM	10 µM	
CYP1A2	100	99.2	96.7	93.8	92.2	89.8	69.5	41.3	7.40
CYP2B6	100	97.5	98.1	90.4	94.2	76.9	47.0	15.2	3.19
CYP2C8	100	102	89.0	98.3	87.8	76.9	25.3	3.87	2.08

CYP2C9	100	99.4	98.2	97.2	95.1	85.1	59.9	29.8	5.01
CYP2C19	100	103	110	101	102	92.0	70.8	40.7	7.31
CYP2D6	100	95.8	98.7	98.6	101	97.0	84.3	59.2	> 10
CYP3A (Midazolam)	100	96.7	97.0	94.5	95.1	95.9	88.2	61.6	> 10
CYP3A (Testosterone)	100	96.6	97.0	94.8	96.3	94.6	82.2	59.9	> 10

^a Percent of control enzyme activity = 100 × (Enzyme activity in the presence of TA / Enzyme activity in the absence of TA). Enzyme activity was calculated from the peak area ratio of CYP probe metabolite to internal standard by LC-MS/MS.

Positive Control Inhibitor	CYP	IC ₅₀ (μM)
α-Naphthoflavone	CYP1A2	0.0164
Thio-TEPA	CYP2B6	4.22
Montelukast	CYP2C8	0.750
Sulfaphenazole	CYP2C9	0.595
(+)-N-3-benzylrivanol	CYP2C19	0.324
Quinidine	CYP2D6	0.0641
Ketoconazole	CYP3A (Midazolam)	0.0450
Ketoconazole	CYP3A (Testosterone)	0.0270

6. APPENDIX 1. ANALYTICAL METHOD

Liquid Chromatography

Column: Waters ACQUITY UPLC BEH Phenyl 30 × 2.1 mm, 1.7 μm

M.P. Buffer: 25 mM ammonium formate buffer, pH 3.5 Aqueous Reservoir (A): 90% water, 10% buffer

Organic Reservoir (B): 90% acetonitrile, 10% buffer Flow Rate: 0.7 mL/minute

Gradient Program:

Time (min)	% A	% B
0.00	90	10
0.50	1	99
0.75	1	99
0.80	90	10
1.00	90	10

Total Run Time: 1.00 minute

Autosampler: 10 L injection volume

Strong Needle Wash: water/methanol/2-propanol:1/1/1; with 0.2% formic acid Weak Needle

Wash: 0.1% formic acid in water

Seal Wash: 10% methanol, 90% water Mass Spectrometer

Instrument: PE SCIEX API 4000

Interface: Turbo Ionspray

Mode: Multiple reaction monitoring

Method: 1.0 minute duration Settings:

Test Article	+/-	Q1	Q3	DP	EP	CE	CXP	IS
NBCoV1	+	470.1	104.9	112	10	47	15	5500

TEM: 500°C; **CAD:** 7; **CUR:** 30; **GS1:** 50; **GS2:** 50

Additional Methods

Enzyme assay. Six NBCoV compounds including four with the higher viral inhibitory activity and 2 with no activity were tested against the 3 enzymes β -lactamase, trypsin, and malate dehydrogenase (MDH). Additionally, the activity of the NBCoV compounds was investigated against the two luciferase enzymes NanoLuc and FLuc, which were used in the single-cycle antiviral assays. For all the assays, the compounds were tested at the higher dose of 2000 nM and less than 0.1% of DMSO was present in each assay.

For the β -lactamase inhibitory assay, we used the colorimetric Beta-Lactamase Inhibitor Screening Kit (Sigma-Aldrich Co. LLC) by following the manufacturer's instructions. Briefly, the β -lactamase was incubated with the NBCoV compounds for 10 minutes at 25 °C before addition of the substrate and the absorbance was measured at 490 nm in Tecan M1000 microplate reader.

For the trypsin inhibitory assay, we used the Trypsin Activity Colorimetric Assay Kit (Sigma-Aldrich Co. LLC) by following the manufacturer's instructions. Trypsin was incubated with the NBCoV compounds for 10 minutes at 25 °C before addition of the substrate and the absorbance was measured at 405 nm.

For the MDH inhibitory assay, we used the colorimetric Malate Dehydrogenase Assay Kit (Sigma-Aldrich Co. LLC) by following the manufacturer's instructions. MDH was incubated with the NBCoV compounds for 10 minutes at 37 °C before addition of the substrate and the absorbance was measured at 450 nm.

For the NanoLuc inhibitory assay, 293T\17 cells were plated at 2×10^4 cells /well in a 96-well tissue culture plate and incubated overnight at 37 °C. The following day, the cells were transfected with 0.1 μ g/well of the pNL4-3 Δ Env-NanoLuc expression vector. Following 24 h incubation, the cells were washed with PBS and lysed with 50 μ L of the cell culture lysis reagent. The lysates were incubated with the NBCoV compounds for 10 minutes at 25 °C. As control, lysates were untreated or treated with the Intracellular TE Nano-Glo® Substrate/Inhibitor (Promega). The lysates were

then transferred to a black plate and mixed with the same volume of Nano-Glo® Luciferase reagent. The luciferase activity was immediately measured with a Tecan SPARK.

For the FLuc inhibitory assay, 293T\17 cells were plated at 2×10^4 cells /well in a 96-well tissue culture plate and incubated overnight at 37 °C. The following day, the cells were transfected with 0.1 µg/well of the pNL4-3.Luc.R-E- expression vector. Following 24 h incubation the cells were washed with PBS and lysed with 50 µL of the cell culture lysis reagent. The lysates were incubated with the NBCoV compounds for 10 minutes at 25 °C. As control, lysates were untreated or treated with 100 µM resveratrol (Sigma-Aldrich Co. LLC). The lysates were then transferred to a white plate and mixed with the same volume of luciferase assay reagent. The luciferase activity was immediately measured with a Tecan SPARK.

Colloidal aggregation study

The cytotoxicity of NBCoV1 small molecule was evaluated in 293T/ACE2 cells in the presence or absence of 0.025% of Tween-80. Briefly, 1×10^4 /well 293T/ACE2 cells were plated in a 96-well cell culture plate and incubated overnight. The following day, aliquots of escalating concentrations of the NBCoV1 were added to the cells and incubated at 37 °C. Following 48 h incubation, the MTS reagent was added to the cells and incubated for 4 h at 37 °C. The absorbance was recorded at 490 nm.

For the infectivity assay in the presence or absence of 0.025% of Tween-80, 293T/ACE2 cells were infected with same amounts of pseudovirus pNL4-3ΔEnv-NanoLuc/SARS-CoV-2. After 48 h incubation, the cells were washed with PBS and lysed with 50 µL of lysis buffer. Twenty-five µL of the cell lysates were transferred to a white plate and mixed with the same volume of Nano-Glo® Luciferase reagent. The luciferase activity was measured immediately with the Tecan SPARK.

The antiviral activity of small molecules NBCoV1 in 293T/ACE2 cells infected with pseudoviruses SARS-CoV-2 was evaluated following the compound's centrifugation. Briefly, a concentrated aliquot of NBCoV1 small molecule in PBS solution was spun at 13000 g for 20 min as previously described (1), and the resulting supernatant was used for the neutralization experiment. For direct comparison, the assay was ran in parallel by using NBCov1 which was not spun. Briefly, 293T/ACE2 cells were plated at 1×10^4 /well in 96-well plates and incubated overnight. On the following day, aliquots of the pseudovirus at about 1500 TCID₅₀/well at an MOI of 0.1 were pretreated with graded concentrations of the NBCoV1-centrifuged or not (NBCoV1-control) for 30 min and added to the cells. 293T/ACE2 cells cultured with medium with or without the SARS-CoV-2 pseudovirus, were included as positive and negative controls, respectively. After 48 h incubation, the cells were washed with PBS and lysed with 50 μ L of lysis buffer. Twenty-five μ L of the cell lysates were processed as above to measure the luciferase activity and calculate the percent inhibition by the NBCoV small molecules and the IC₅₀ values.

Table S1. Antiviral activity of NBCoV small molecules against pseudovirus NL4-3ΔEnv-NanoLuc/SARS-CoV-2 (IC₅₀) in single-cycle assay in 293T/ACE2 cells (cells were pre-treated with the NBCoV compounds for 30 min before infection) and antiviral activity of NBCoV small molecules against the replication-competent authentic virus SARS-CoV-2 (US_WA-1/2020) virus (IC₁₀₀) in Vero E6 cells (cells were pre-treated for 2 h before infection).

Compound	Pre-treated 293T/ACE2 cells/ SARS-CoV-2 IC ₅₀ (nM)	Pre-treated Vero cells/ SARS-CoV-2 IC ₁₀₀ (μM)
NBCoV1	>2000	>10
NBCoV2	>2000	>10
NBCoV3	>2000	>10
NBCoV4	>2000	>10
NBCoV5	>2000	>10
NBCoV6	>2000	>10
NBCoV7	>2000	>10
NBCoV8	>2000	>10
NBCoV9	>2000	>10

Table S2. Evaluation of NBCoV small molecules against β-lactamase, Trypsin, and MDH

Compound	β-lactamase IC ₅₀ (nM)	Trypsin IC ₅₀ (nM)	MDH IC ₅₀ (nM)
NBCoV1	>2000	>2000	>2000
NBCoV2	>2000	>2000	>2000
NBCoV3	>2000	>2000	>2000
NBCoV4	>2000	>2000	>2000
NBCoV5	>2000	>2000	>2000
NBCoV34	>2000	>2000	>2000

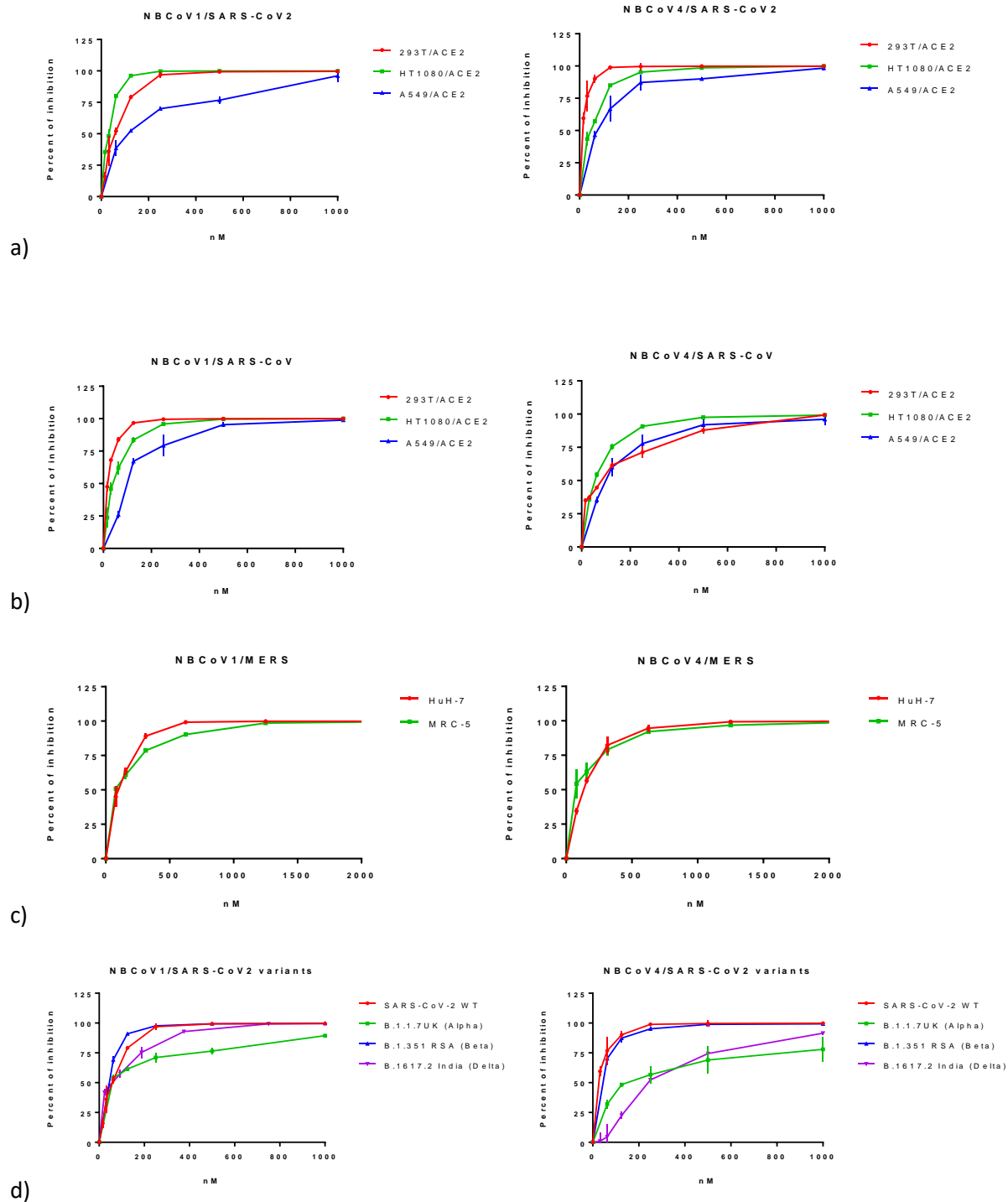


Figure S1. Representative dose-response curves of the antiviral activity of NBCoV1 and NCoV4 in single-cycle assay in different cell lines against pseudoviruses **a)** NL4-3ΔEnv-NanoLuc/SARS-CoV-2; **b)** NL4-3ΔEnv-NanoLuc/SARS-CoV; **c)** NL4-3ΔEnv-NanoLuc/MERS-CoV; **d)** Representative dose-response curves of the antiviral activity of NBCoV1 and NCoV4 in single-cycle assay in 293T/ACE2 cells against NL4-3ΔEnv-NanoLuc/SARS-CoV-2 mutant pseudoviruses variants B.1.1.7 UK (Alpha), B.1.351 RSA (Beta) and B.1.617.2 India (Delta). Values represent the means \pm standard deviations ($n = 3$)

TMPRSS expression

Cell lysates were immunodetected using the TMPRSS2 Recombinant Rabbit Monoclonal Antibody (ARC1439) (Invitrogen) and with the anti- β -Actin mAb (Sigma) which was used as a loading control. The intensity of the bands was quantified using ImageJ software (<http://imagej.nih.gov/ij/>)

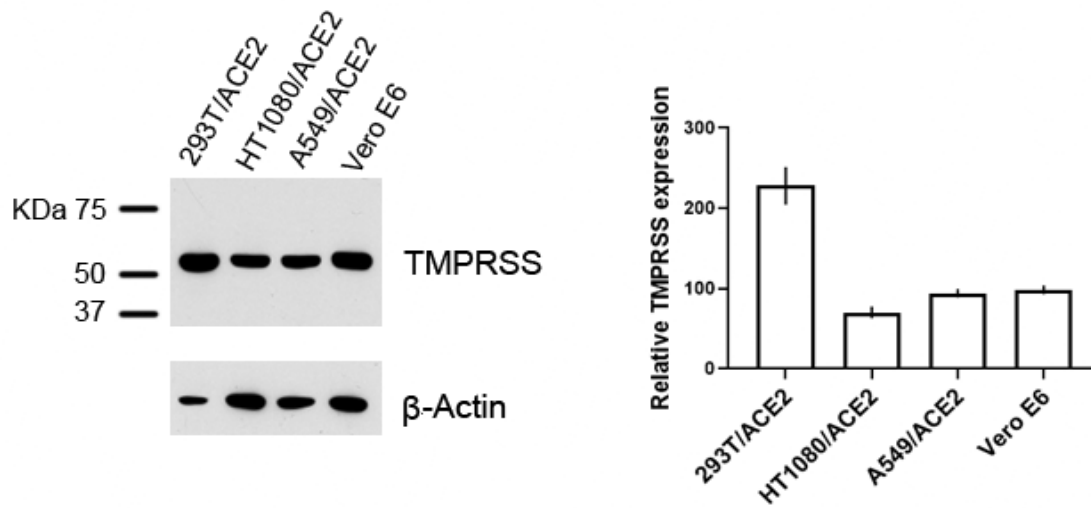


Figure S2. Evaluation of TMPRSS expression and related densitometry analysis in different cell lines. Cell lysates were immunodetected using the TMPRSS2 mAb. The densitometry analysis of the TMPRSS is relative to β -actin expression which was used as a loading control. Immunoblots are representative of two independent experiments. Columns represent the means \pm standard deviations (n=2).

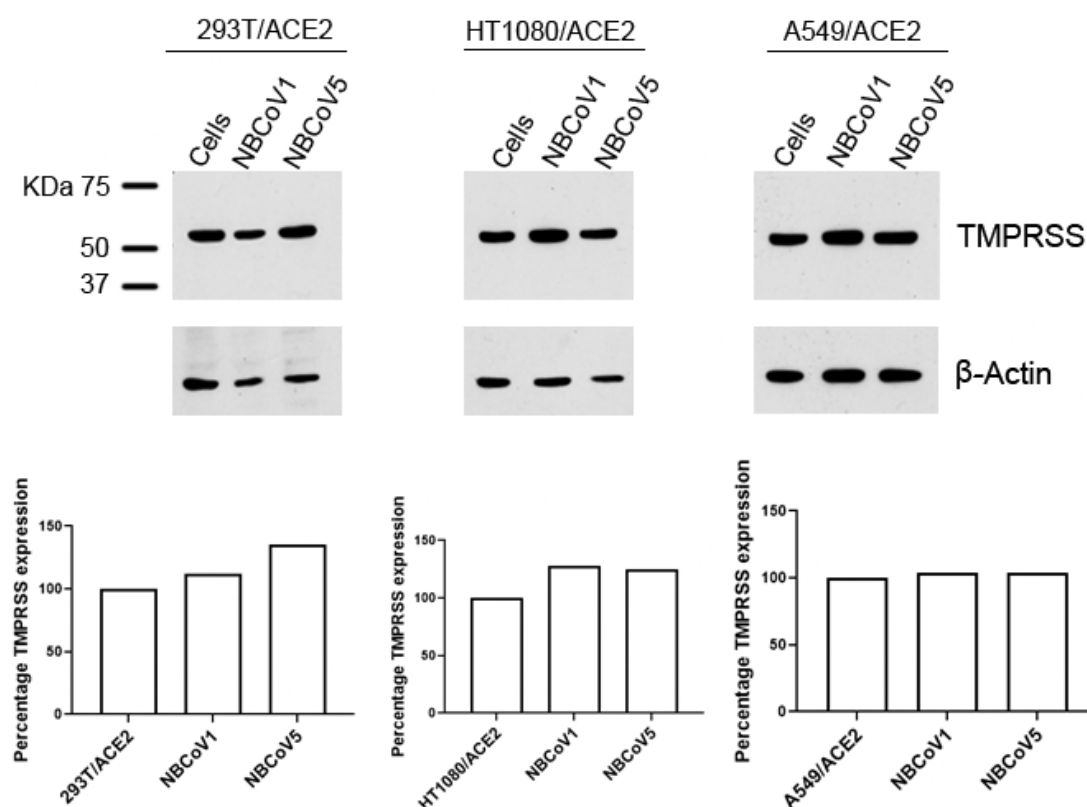


Figure S3. Evaluation of TMPRSS expression and related densitometry analysis in different cell lines. Cell were untreated or treated with 1 μ M of either NB-CoV1 or NB-CoV5 for 48 hr. Cell lysates were immunodetected using the TMPRSS2 mAb. The densitometry analysis of the TMPRSS is relative to β -actin expression, which was used as a loading control, and expressed as percentage respect to the respective untreated controls. Immunoblots are representative of two independent experiments. Columns represent the means \pm standard deviations (n=2).

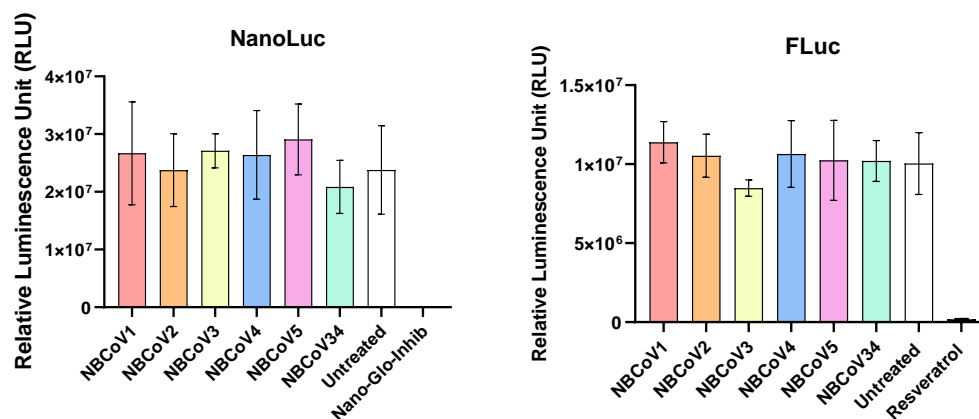


Figure S4. Evaluation of NBCoV small molecules against NanoLuc and FLuc enzymes. Lysates of 293T\17 cells expressing a) NanoLuc or b) FLuc were incubated with the NBCoV compounds for 10 minutes at 25 °C. As control, lysates were untreated or treated with the Intracellular TE Nano-Glo® Substrate/Inhibitor a) or with 100 µM of Resveratrol b). The lysates were then, mixed with the same volume of the respective substrate. Columns represent the means \pm standard deviations (n = 2-3)

The molecule with SMILES

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OC(=O)C1=CC(=CC=C1Cl)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
OC(=O)C1=CC(=CC=C1)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
OC(=O)C1=CC(=CC=C1F)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
CC1=CC=C(C=C1C(O)=O)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
CC1=C(C=CC=C1C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O)C(O)=O
CCC1=CC=C(C=C1C(O)=O)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
OC(=O)C1=C(F)C(=CC=C1)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
OC(=O)C1=CC(=CC=C1Br)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
CC1=C(C=NC=C1C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O)C(O)=O
ClC1=CC=C(C=C1)C1=CC=C(O1)\C=C1/SC(=S)N(CCC2=CC=CC=C2)C1=O
S=C(S/1)NC(C1=C\C(O2)=CC=C2C3=CC=C(Cl)C(C(O)=O)=C3)=O
S=C(S/1)NC(C1=C\C(O2)=CC=C2C3=CC=C(C(O)=O)C(Cl)=C3)=O
S=C(S/1)N(CC#C)C(C1=C\C(O2)=CC=C2C3=CC=C(Cl)C(C(O)=O)=C3)=O
S=C(S/1)NC(C1=C\C(O2)=CC=C2C3=CC=C(C(O)=O)C=C3)=O

```

was not similar to any known aggregator in our database.

However, since this molecule has a fairly high calculated LogP of **5.1** which is in the range reported for many other aggregators, we strongly urge you to perform appropriate controls to test for possible aggregation. Click [here](#) for guidance on how to perform these tests.

Created by Gurgen Tumanian, Allison Doak, Teague Sterling, John Irwin and Brian Shoichet with assistance from Pascal Wassam. We thank [NIGMS](#) for financial support. [top](#)

Figure S5. The data from Aggregator Advisor run could not find any known aggregator with similar structure.

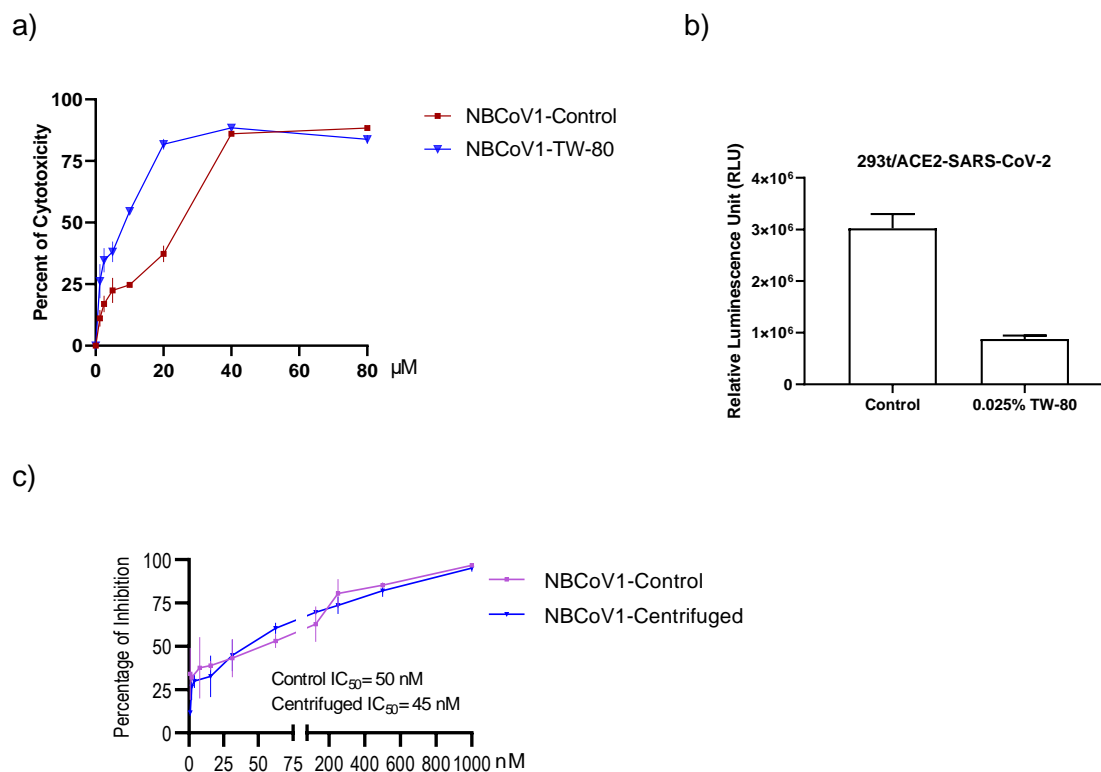


Figure S6. Colloidal-aggregation studies with NBCoV1. a) Cytotoxicity assay of NBCoV1 performed in 293T/ACE2 cells in the presence or absence of 0.025% of Tween-80 (the values represent the means \pm standard deviations ($n = 3$)). b) Infection of 293T/ACE2 cells with same amounts of pseudovirus SARS-CoV-2 in the presence or absence of 0.025% of Tween-80 (control). Columns represent the means \pm standard deviations ($n = 3$). c) Neutralization assay with NBCoV1-centrifuged and not-centrifuged (NBCoV1-control) in 293T/ACE2 cells infected with pseudovirus SARS-CoV-2 (the values represent the means \pm standard deviations ($n = 3$)).

REFERENCES

1. Sassano MF, Doak AK, Roth BL, Shoichet BK. 2013. Colloidal aggregation causes inhibition of G protein-coupled receptors. *J Med Chem* 56:2406-14.