EMORY | CHEMICAL BIOLOGY DISCOVERY CENTER

MLSCN Probe Report

Project Title: High Throughput Screening-based Identification of Measles Virus Probes

Grant Number: American Lung Association, AI056179 & AI1071002 (NIH/NIAID) and U54 HG003918 (NIH)

Screening Center Name: Emory Chemical Biology Discovery Center

Principal Investigator of Screening Center: Raymond J. Dingledine

Assay Submitter & Institution: Richard K. Plemper and James P. Snyder, Emory University

Assay or Pathway Target: Measles Virus

Probe PubChem Compound Identifier (CID): 16122506

Assay provider information

Specific aim 1: *to identify novel MV-specific probes through high throughput screening (HTS) of the MLSCN library* ***(primary assay)***
We hypothesize that HTS will identify hits that are structurally and mechanistically distinct from our existing fusion protein-specific probes. We will provide an automated cell-based assay that monitors a fluorescent MV reporter to identify probe candidates. If necessary, assay parameters will be optimized in collaboration with MLSCN. We will reassess all candidates in the same assay to ensure data reproducibility.
Rationale/Aim 1. The discovery of small molecule probes that target different stages of the MV life cycle is imperative for the ultimate development of novel therapeutics against MV. We will provide a cell-based automated assay for identification of hits that target different early stages in the viral life cycle such as receptor binding, membrane fusion, and viral polymerase function. It is a particular advantage of our assay strategy that different probes with diverse target spectrums can be discovered in parallel. This greatly increases the likelihood to identify promising hits, extracts a maximum of information from a single screen, and may open future avenues to circumvent viral resistance to individual inhibitors emerging in the field.

Specific aim 2: *to counterscreen putative probes in independent assays and rank confirmed hits by active concentration and cytotoxicity* ***(secondary confirmatory assays)****.*
We hypothesize that HTS will generate a certain amount of false-positive hits, necessitating counterscreening of all identified candidates in secondary assays. We will subject all potential hits to three independent manual assays for confirmation and assessment of inherent cytotoxicity. Full dose-response curves will be generated from these assays and 50% effective (EC_{50}) and cytotoxic (CC_{50}) concentrations calculated for all confirmed hits. We will rank inhibitor candidates by activity and selectivity index (CC_{50}/EC_{50}).
Rationale/Aim 2: Automated screening necessitates secondary assays with different readouts to confirm putative hits and eliminate false-positive results. We have therefore developed three independent assays to confirm hits and quantify cytotoxicity and active concentrations. All putative hits will be subjected to these assays and a small panel of confirmed probes with high specific activity will be identified.

Specific aim 3: *to determine the specificity of selected probes and assess their mechanisms of antiviral activity* ***(initial probe characterization)****.*
We hypothesize that a panel of distinct compounds with diverse target spectrums will provide further insight into host pathogen interaction and open future avenues to counteract resistance that may emerge to individual inhibitors in the field. To determine specificity of new probes, activity against a panel of related members of the paramyxovirus family and clinically relevant primary MV isolates will first be assessed. Highly potent compounds will then be subjected to mechanistic classification of antiviral activity. In separate assays, we will examine their effect on receptor binding, viral entry, and viral gene expression and genome replication.
Rationale/Aim 3: Towards generating highly selective probes that constitute a valuable platform for the future development of novel therapeutics, high target specificity of candidate compounds is imperative. The activity of highly promiscuous compounds is likely based on indirect mechanisms or, in the case of candidate antivirals, on underlying cytotoxicity, which contributes to the antiviral effect. Early evaluation of target specificity is therefore required. A subset of the most potent and target specific hit compounds will then be subjected to initial mechanistic characterization. This procedure will ensure that a panel of candidate probes with high selectivity and a diverse target spectrum will be identified for the follow-up research program.

Significance: Despite the existence of a live-attenuated vaccine, MV remains one of the major human pathogens, causing significant morbidity and mortality worldwide. The identification of novel small molecule probes that target different early stages of the viral life cycle will be instrumental for an improved mechanistic understanding of the infectious cycle and ultimately the development of novel intervention strategies. Conceptually, MV-based findings will likely be transferable to other clinically relevant members of the paramyxovirus family, preparing the path for a better mechanistic understanding of these pathogens and the future development of antivirals directed against related established and emerging paramyxoviruses for which no vaccine is currently available.

Screening center information
Assay Implementation and Screening
PubChem Bioassay Name: Non-Nucleoside Inhibitor of Measles Virus RNA-Dependent RNA Polymerase Complex Activity HTS Single Point

List of PubChem bioassay identifiers generated for this screening project (AIDS):
745, 815

Primary Assay Description as defined in PubChem:
Vero cells were seeded in 96-well microtiter plates at a density of 7,500 cells per well in 100 μl growth medium. After a four-hour incubation period at 37°C and 5% CO2, compound was added in 1.0 μl/well doses (25 μM final concentration) with a Sciclone automated liquid handler system (Caliper, MA), followed by infection with rMV-eGFP at an MOI of 0.25 pfu/cell in 100 μl serum-free medium. Final solvent (DMSO) concentrations were 0.5% at which no adverse effect on cell viability or virus growth could be detected in control samples. All virus stocks used for screening were subjected to dialysis against PBS to remove contaminating eGFP that has been synthesized during virus growth. Following a 64-hour incubation period at 37°C, green fluorescence indicating expression of virus-encoded eGFP was quantified using an Analyst HT microplate reader (Molecular Devices). To validate the assay, the MV fusion inhibitor AS-48 was added in an otherwise identical setting and z' values were calculated according to the formula:

z' = 1- (3SD(C)+3SD(B))/(Mean(C)-Mean(B)), with C: control and B: background.

As first-pass test to exclude false-positive compounds, cytotoxicity was assessed microscopically for all wells that showed low fluorescence intensity and, for selected compounds, photo-documented at a magnification of 200x. The compound library used is a diversity set purchased by the Emory Chemical Biology Discovery Center from ChemDiv (San Diego, CA). Data were normalized for deposition into PubChem using the number of standard deviations from the mean compound value per plate. The field SDs from Mean was calculated by the formula:

Y = (CompoundFluorescence - MeanCompoundFluorescence) / SDCompoundFluorescence, where the mean and sd refer to the aggregate value for all compound wells on a plate.

PUBCHEM_ACTIVITY_SCORE was obtained by normalizing the number of SDs from the mean between the highest observed value for the assay and the lowest observed value, according to the formula:

SCORE = 100 * (Value - MaxVal) / (MinVal - MaxVal)

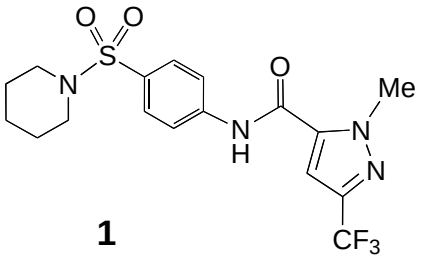
Center Summary of the Primary Screen:

Optimized assay stats			Library	# screened	# hits	Hit %
Plate format	Mean Z'	Mean S:B				
96	0.8	6.43	ChemDiv (Emory collection)	33,360	41	0.12

Probe Optimization

Chemical name of probe compound:
2-methyl-N-(4-piperidin-1-ylsulfonylphenyl)-5-(trifluoromethyl)pyrazole-3-carboxamide

Draw probe chemical structure and show stereochemistry if known:



1

This structure will be referred to as compound **1** below. The substance is neither chiral nor does it contain any special stereochemical features that are not pictured above.

Describe mode of action for biological activity of probe:
The compound represents a first-in-class non-nucleoside inhibitor of MV RNA-dependent RNA polymerase complex activity.

Has this compound been provided to the MLSMR: No

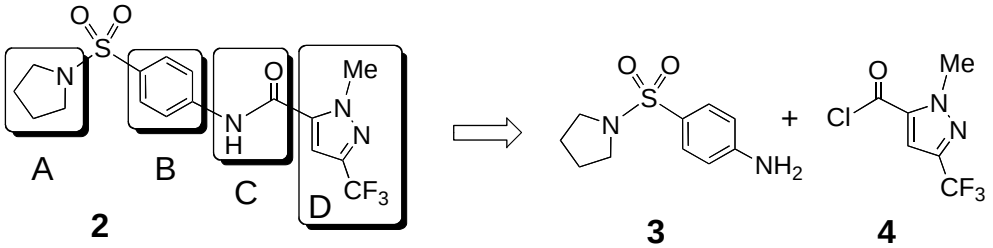
Description of secondary screens used to optimize probe structure:[i]

A) Dose-response assays: Two separate assays – based on suppression of virus-induced cytopathicity or reduction of virus yields - were used to assess the potency of candidate compounds. For the former assay, cells were infected in four replicates per compound concentration in a 96-well plate format at a multiplicity of infection of 0.2 pfu/cell in the presence of compound in two-fold dilutions with starting concentrations of 75 μM. At

96 hours post-infection, virus-induced cytopathicity was determined through staining of plates with 0.2% crystal violet and measuring of absorption at 540 nm in a microplate reader. Values were calculated according to the formula [% rel. CPE = 100-(experimental-background)/(maximum-background)*100]. For the second assay, cells were infected in a 6-well plate format at a multiplicity of infection of 0.1 pfu/cell in the presence of compound in ten-fold dilutions with starting concentrations of 100 μM, and incubated in the presence of compound at 37ºC for 30 hours. Cell associated viral particles were then harvested and virus titers determined by $TCID_{50}$ titration.

B) Cytotoxicity assays: Two assays, a non-radioactive cytotoxicity assay (CytoTox 96 Non-Radioactive Cytotoxicity Assay, Promega) and a trypan-blue exclusion assay, were employed to determine cytotoxicity of compounds. For the cytotoxicity assay, 12,000 cells per well in a 96-well plate format were incubated at 37ºC for 24 hours in four replicates per concentration tested in the presence of a range of compound concentrations in 2-fold dilutions (300 μM highest). Conversion of a tetrazolium salt (INT) into a colored formazan product by cellular lactate dehydrogenase released into the culture supernatants was then measured at 490 nm using a BioRad plate reader. Values were calculated according to the formula [% viability = 100-((experimental-background)/(maximum-background)*100)]. For the trypan-blue exclusion assay, $2x10^5$ cells per well were seeded in a 6-well plate format and incubated at 37ºC for 30 hours in three replicates per concentration tested over a 5-fold dilution range (500 μM highest). Cells were then detached from culture dishes, aliquots incubated with trypan-blue solution for 15 minutes at room temperature, and the number of viable cells counted using a hemacytometer.

Center comments on chemistry strategy leading to probe identification:



The initial lead compound from HTS in the present series was **2**. It proved highly active against multiple primary isolates of diverse MV genotypes currently circulating worldwide, showing active concentrations of 35 to 145 nM.[i] In attempts to improve activity, the structure was divided into the four sectors labeled A-D. A wide variety of analogs in sectors B-D were prepared by the retrosynthetic strategy indicated by the disconnection to fragments **3** and **4**. To our surprise, none of these compounds proved to be superior to the hit compound. Lastly, synthesis turned to replacement of the pyrrolidine ring in sector A. The piperidine ring replacement

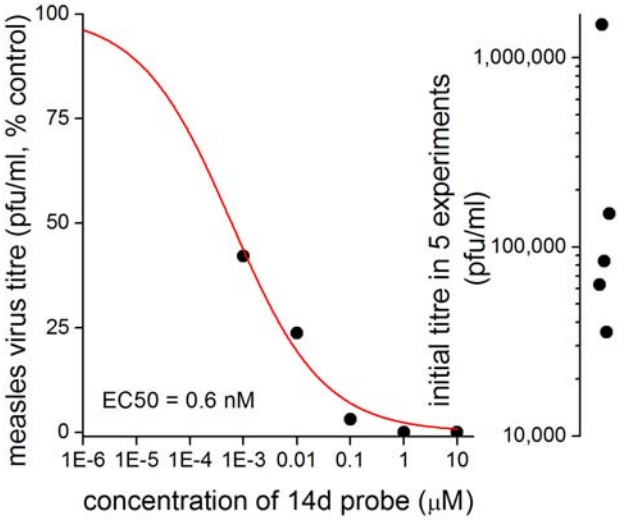
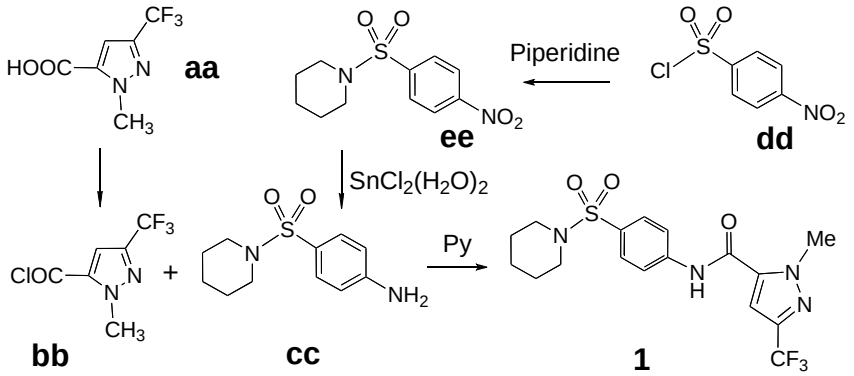


Figure 1. Dose-response curve for the action of compound **1** as determined by $TCID_{50}$ titration in the virus titer assay. Both % control and raw virus titre data are shown. Data shown are n = 1, representative of 5 independent experiments (mean EC_{50} = 3.8 nM).

pictured above as probe 1 delivers activity at an EC_{50} = 3.8 nM (95% confidence interval 0.11 nM – 130 nM) against live measles virus (strain Alaska, cf. **Figure 1**). See SAR discussion below.

Detailed synthetic pathway for making probe:



Compound 1.[v,vi] 1-Methyl-3-trifluoromethyl-5-pyrazolecarboxylic acid **aa** (4 mmol, 776 mg) was prepared as previously described,[ii] dissolved in dichloromethane (10 ml) and treated with oxalyl chloride (2 eq, 2.0 M in CH_2Cl_2) and a catalytic amount of DMF (2 drops). The reaction mixture was kept at room temperature for 5 h. Solvent removal by evaporation delivered yellow acyl chloride **bb** in quantitative yield. A portion of the latter in dichloromethane (0.55 mmol) was added dropwise to an ice-cold solution of 4-amino-prolidinyl sulfonamide **cc**[iii] (120.2 mg, 0.5 mmol) and pyridine (48 microl, 0.6 mmol) in dichloromethane (2 ml). The reaction mixture was allowed to warm to room temperature slowly with stirring for an additional 3 h, poured into dilute hydrochloric acid (1N), extracted with dichloromethane (3x15 ml) and dried over anhydrous Na_2SO_4. The product was purified by silica gel flash chromatography using hexane/ethyl acetate (3:1) to obtain compound **1** as a white powder (126.9 mg, 61% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43-1.44(m, 2H), 1.63-1.66(m, 4H), 3.0(t, 2H, J=3.6Hz), 4.28(s, 3H), 6.98(s, 1H), 7.75 (d, 1H, J=6.0Hz), 7.78 (d, 1H, J=6.0Hz), 7.84 (s, 1H). Anal. Calcd for $C_{17}H_{19}F_3N_4O_3S$: C, 49.03; H, 4.60 ; N, 13.45. Found: C, 49.14; H, 4.64; N, 13.40.

General procedures for synthesis of 4-amino-sulfonamide cc.
Pyridine (11mmol, 1.1eq) was added dropwise to a mixture of 4-nitro-benzenesulfonyl chloride **dd** (10.0mmol, 2.22g) and piperidine (11mmol, 1.1eq) in dichloromethane (20ml) at 0°C. The reaction was gradually warmed to room temperature and stirred for 10h, poured into dilute HCl (1N), extracted with dichloromethane (3x15ml) and dried over anhydrous Na_2SO_4. Solvent was evaporated to obtain the corresponding 4-nitro-sulfonamide **ee**, which was subjected to reduction without further purification. Thus, **ee** (1.0mmol) in ethyl acetate was treated with tin chloride dihydrate (5.0mmol, 5.0 eq) at room temperature for over 8h until TLC reveals the absence of starting material. The reaction mixture was poured into $NaHCO_3$ (sat.), extracted with ethyl acetate (3x20ml), and the product purified by chromatography.

Known probe properties

Cell Toxicity was determined by two methods:[i] MTT cytotoxity assay and a Trypan blue exclusion assay. Based on the EC_{50} = 12 uM (MV-Alaska) for virus titer reduction, the corresponding selectivity indices SI(CC_{50}/EC_{50}) are $\geq$ 130 and 14,200, respectively.

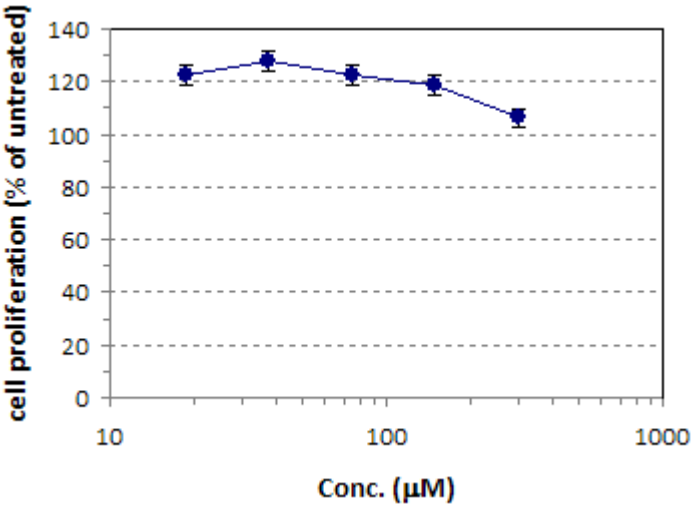


Figure 2. Dose-response curve for the cytotoxicity of compound **1** as determined by the trypan-blue exclusion assay. Data are mean SEM, n = 3 independent experiments.

Reactivity* and *Physical Properties No significant properties have been measured for 1, but a selected variety of such properties have been calculated using the QikProp software.[iv] None of the values of the properties calculated fall outside the 95% range of similar values for known drugs.

Reactivity Compound **1** is completely stable under ambient conditions and as a solution in DMSO in the cold. Since the molecule has no reactive functional groups, it would appear to be free of decomposition/reactivity/ toxicity problems *in vivo*.

QikProp Predictions:
CNS activity: -1 on a scale of -2 to +2
Dipole moment: 5.4 D
SASA (total solvent accessible surfaced area; probe radius 1.4 Å): 681 $Å^2$
Volume (total solvent accessible volume; probe radius 1.4 Å): 1193 $Å^3$
QPlogPo/w(Predicted water/gas partition coefficient): 3.2
QPlogS (Predicted aqueous solubility, log S. S in moles/liter is the concentration of the solute in a saturated solution that is in equilibrium with the crystalline solid): -5.6
QPlogHERG: -5.5
QPPCaco (apparent Caco-2 cell permeability in nm/sec): 638
QPPMDCK (apparent MDCK cell permeability in nm/sec): 1482
QPlogBB (Predicted brain/blood partition coefficient): -0.68
*QPlog*Kp *(Predicted skin permeability):* -2.9
HumanOralAbsorption: 2
%HumanOralAbsorption: 77

SAR around Probe 1

At present, around 100 analogs of the hit compound have been prepared and assayed in both primary and secondary screens.[v,vi] From a broad SAR perspective, the current series seems to require maintenance of the molecular elements pictured in sectors B-D of **2**. By contrast, a wide range of hydrophobic alterations of the amine in sector A are tolerated to provide low nM activity against the virus. A subset of the compound activities illustrate the main SAR trends in **Tables 1-3** (also provided in Ref. 5). The chemical structures are provided in Ref. v.

Table 1. MV antiviral EC_{50}s and CC_{50}s for *N*-[4-(pyrrolidinylsulfonyl)phenyl]-1H-phenyl amides; aromatic ring replacing the pyrazole ring in Sector D of **2**.

ID	Comp.	EC_{50} (μM)[a] (MV-Alaska)		CC_{50} (μM)[b] (Vero cells)	
3a	MS-8	CPE inhibit	> 75	MTT cytotox	> 300
3b	MS-9	CPE inhibit	> 75	MTT cytotox	> 300
3c	MS-10	CPE inhibit	48± 6.1	MTT cytotox	> 300
3d	AS-36b	CPE inhibit	ND[c]	MTT cytotox	11± 1.3
3e	AS-60a	CPE inhibit	>75	MTT cytotox	> 300
3f	AS-36a	CPE inhibit	ND[c]	MTT cytotox	59 ± 4
3g	AS-27	CPE inhibit	>75	MTT cytotox	> 300
3h	AS-40	CPE inhibit	≥ 75	MTT cytotox	76 ± 3
3i	AS-60b	CPE inhibit	>75	MTT cytotox	> 300
3j	AS-60d	CPE inhibit	>75	MTT cytotox	> 300
3k	AS-61d	CPE inhibit	>75	MTT cytotox	> 300
3l	AS-57a	CPE inhibit	>75	MTT cytotox	> 300
3m	AS-57b	CPE inhibit	>75	MTT cytotox	> 300
3n	MS-13	CPE inhibit	> 75	MTT cytotox	> 300
3o	MS-30	CPE inhibit	> 75	MTT cytotox	> 300
3p	MS-11	CPE inhibit	> 75	MTT cytotox	> 300
3q	MS-17	CPE inhibit	25	MTT cytotox	140
3r	AS-119	CPE inhibit	>75	MTT cytotox	37 ± 1.5
3s	MS-12	CPE inhibit	65	MTT cytotox	>300

a: Values represent averages of four experiments ± SD; highest concentration assessed 75 μM.
b: Values represent averages of two experiments ± SEM; highest concentration assessed 300 μM.
c: EC_{50} not determined (ND) when CC_{50} ≤75 μM.

Table 2. MV antiviral EC_{50}s and CC_{50}s for substituted phenyl rings and various linkers in sectors B and C of **2**.

ID	Comp.	EC_{50} (μM)[a] (MV-Alaska)		CC_{50} (μM)[b] (Vero cells)	
4a	MS-37	CPE inhibit	> 75	MTT cytotox	288 ± 13
4b	MS-14	CPE inhibit	≥ 75	MTT cytotox	> 300
4c	MS-38	CPE inhibit	ND[c]	MTT cytotox	14 ± 0.5
4d	MS-15	CPE inhibit	ND[c]	MTT cytotox	13 ± 0.3
5	MS-26	CPE inhibit	15.5 ± 2.4	MTT cytotox	> 300
6	MS-27	CPE inhibit	41 ± 39	MTT cytotox	> 300
7	MS-34	CPE inhibit	> 75	MTT cytotox	> 300
8	MS 36	CPE inhibit	> 75	MTT cytotox	> 300

a: Values represent averages of four experiments ± SD; highest concentration assessed 75 μM.
b: Values represent averages of two experiments ± SEM; highest concentration assessed 300 μM.
c: EC_{50} not determined (ND) when CC_{50} ≤ 75 μM.

Table 3. MV antiviral EC_{50}s and CC_{50}s of 1-Methyl-3-(trifluoromethyl)-N-[4-(pyrrolidinylsulfonyl)-phenyl]-1H-heterocyclic ring-5-carboxamides; replacements of the pyrrolidine ring in sector A of **2**.

ID	Comp.	EC_{50} (μM)[a] (MV-Alaska)		CC_{50} (μM)[b] (Vero cells)	
14a	AS-85a	CPE inhibit	14 ± 2	MTT cytotox	100
14b	AS-105	CPE inhibit	23 ± 10	MTT cytotox	> 300
14c	AS-103	CPE inhibit	ND[c]	MTT cytotox	13 ± 0.7
14d	AS-136a	CPE inhibit	<2.3	MTT cytotox	>300
14e	AS-251	CPE inhibit	>75	MTT cytotox	> 300
14f	AS-244	CPE inhibit	28 ± 9	MTT cytotox	126 ± 7
14g	AS-236	CPE inhibit	43 ± 24	MTT cytotox	> 300

a: Values represent averages of four experiments ± SD; highest concentration assessed 75 μM
b: values represent averages of two experiments ± SEM; highest concentration assessed 300 μM
c: EC_{50} not determined (ND) when CC_{50} ≤75 μM

Recommendations for the scientific use of probe as a research tool: The probe described above is a selective, first-in-class inhibitor of the measles virus RNA-dependent RNA polymerase complex. It has shown *in vitro* efficacy against 8 primary MV isolates as well as *in vivo* efficacy in rats against intranasal MV infection. The primary research utility of this probe will be to elucidate the mechanisms of the measles virus infectious cycle as well as the development of novel intervention strategies and combination therapies. The probe may also be used with other clinically relevant members of the paramyxovirus family for which no vaccine is currently available.

Second Screen

The above work resulted from a 96-well plate HTS screen of 34,000 compounds from the ChemDiv compound library located at Emory. Subsequently, we have also screened the MLSMR collection (139,000 compounds) in a 384-well plate format using a slightly modified assay. Although the primary screening is still underway, the hit rate appears to be ~0.4 compounds per 1,000 compounds screened (~0.04%). Many of the hits are expected to be RNA-dependent RNA polymerase inhibitors and, thus, not different in mechanism to compound **1** as a probe. However, secondary assays will evaluate if an additional and alternative target can provide a second, independent probe against the virus.

Appendices

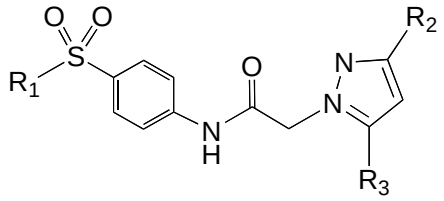
Comparative data on probe, similar compound structures and prior probes

PubChem has been searched for compounds with >90% similarity with probe **1**. Two classes of compounds, one of which is identical with the probe were found. **Table 4** below summarizes the structures and biological data.

Table 4. Nine PubChem structures that are 90% similar to the MV probe **1** (CID 16122506)

CID	R_1	R_2	R_3	Activity
1225548	NHPh(2-Me)	Me	CF_3	No data
1236028	[a]			No data
2814183	NH_2	Me	*t*-Bu	No data
3243581	NHEt	Me	CF_3	inactive 135 assays
3244508	[a]			inactive CYP2C9
4397834	pyrrolidine	Me	CF_3	Inactive in two assays[b,c]
5011592	NH_2	Et	H	No data
6468662	NMe_2	Me	H	No data
6470230	Pip (p-Me)	Me	H	No data
16122506	Probe **1**	Me	CF_3	No data

[a] Two compound structures are shown below.
[b] Inactive in the 14-3-3 assay and the single point primary assay for detecting MV blockers.
[c] In a manual microscopic follow-up to the primary assay, this compound was found to be active at 35-145 nM as described in the text, serving as the lead to probe **1**.



CID 1236028: R_1 NHMe, R_2 Me, R_3 CHF_2
CID 3244508: R_1 NHMe, R_2 Me, R_3 CF_3

The activity data is incomplete. More detailed work for CIDs 16122506 and 4397834 can be found in Refs v and vi.

Comparative data showing probe specificity for target

To date, the only specificity data concerns strains of the measles virus and closely related paramyxoviruses of the same genus (i.e. canine distemper virus); Ref i.

Also see submitted Manuscript; Ref vi.

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[i] White, L. K.; Yoon, J.-J.; Lee, J. K.; Sun, A.; Du. Y.; Fu, H.; Snyder, J. P.; Plemper, R. K. Nonnucleoside Inhibitor of Measles Virus RNA-Dependent RNA Polymerase Complex Activity. *Antimic. Agents Chemother.* **2007**, *51*, 2293-2303.

[ii] Schlosser, M.; Volle, J.; Leroux, F.; Schenk, K. Switchable Reactivity: The Site-Selective Functionalization of Trifluoromethyl-Substituted Pyrazoles.*Eur. J. Org. Chem*. **2002**, 2913-2920.

[iii] Reaction of 4-nitro-benzene sulfonyl chloride with pyrrolidine in the presence of pyridine afforded 4-nitro-prolidinyl sulfonamide. Reduction with tin dichloride dihydrate in ethyl acetate provided 4-amino-prolidinyl sulfonamide **5** in excellent yield (90%).

[iv] Jorgensen, W. L. The Many Roles of Computation in Drug Discovery. *Science* **2004**, *303*, 1813-1818.

[v] Sun, A.; Chandrakumar, N.; Yoon, J.-J.; Plemper, R. K.; Snyder, J. P. Nonnucleoside inhibitors of the measles virus RNA-Dependent RNA polymerase activity: Synthesis and in vitro evaluation. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5199-5203.

[vi] Sun, A.; Yoon, J.-J.; Prussia, A.; Yin, Y.; Yang, Y.; Plemper, R. K.; Snyder, J. P. Potent Non-Nucleoside Inhibitors of the Measles Virus RNA-Dependent RNA Polymerase Complex. *J. Med. Chem.* **2007**, submitted.