Anantpadma et al. **"Large Scale Screening and Identification of Novel Ebolavirus and Marburgvirus Entry Inhibitors"**

Supplementary Materials:

Methods:

Production of MARVGP-VSVluc.

For preparing MARVGP-VSVluc, 293FT cells grown in 10 cm^2 tissue culture dishes were transfected with 10 µg of plasmid expressing MARV-GP using calcium phosphate method. Two days post transfection cells were inoculated with a VSV seed stock. This stock was derived from a recombinant VSV with a deletion of VSV-G and insertion of firefly luciferase encoding gene, this was a kind gift of Dr. Sean Whelan from Harvard Medical School. It had been pseudotyped to bear the GP of Venezuelan equine encephalitis virus. This was useful as it reached high titer and the GP degraded quickly upon inoculation of cells. One day post infection, culture supernatants containing MARV GP pseudotyped viruses were harvested and tested for infection by measuring expression of firefly luciferase in 293FT cells.

Production of replication-defective MLV pseudotyped with MARV GP.

Replication defective MLV, capable of a single round of infection to express a red fluorescent protein (pStrawberry), pseudotyped with MARV GP was produced. Briefly, 293T cells grown in 10 cm^2 tissue culture dishes were transfected with 5µg each of plasmids encoding MLV GAG-Pol, pFB-strawberry and MARV (Musoke) GP (15 µg total) using calcium phosphate method. One day post transfection the cells were washed once with medium containing no FBS or antibiotics and then overlaid with fresh complete medium. Two days post transfection the supernatant from

transfected cells were collected, filtered through a 0.45 micron filter (to filter cell debris) and stored at -80°C for further use.

Primary screening using MARVGP-VSVluc.

Two µL of 293FT cell suspension were dispensed at 1000 cells/well into solid white 1536-well plates (Grenier) using a Multidrop Combi (Thermo Scientific). Following an overnight incubation at 37°C and 5% CO_2, 23 nL compound was transferred into each well using a pin tool (Kalypsys). The plate was incubated for 1 h at 37°C and 5% CO_2, then 3 µL MARVGP-VSVluc at a 1:100 dilution was added. After 1 d of incubation at 37°C, 5% CO_2, 2 µL of luciferase detection reagent (prepared from D-Luciferin, Potassium salt powder, Goldbio, Cat#LUCK-100) was added. Plates were incubated for 15 min at ambient temperature and then read using a ViewLux plate reader (Perkin Elmer) to detect luminescence. Assays were performed in sub-saturating amounts of virus (multiplicity of infection-MOI <0.5), therefore luciferase signals reflect the amount (titer) of virus able to infect the cells in presence of the compound.

Data was normalized to the median of the positive control (Bafilomycin) and DMSO only wells. Four-parameter logistic dose response curves were fitted to the data using a custom grid based algorithm(3). The fitting procedure produced IC_{50} (concentration of half-maximal inhibition of virus infection) and efficacy (maximal response) values. Fitted curves were also assigned a curve class used to assess the dose response curve quality. Briefly, complete dose response curves with efficacy > 80% and well-defined upper and lower asymptotes were classified as 1.1 curves and are considered the best quality. Class 2.1 and 2.2 were incomplete curves having only one asymptote, with efficacy of 80% or higher and lower than 80%, respectively. Class 3 curves showed activity at only the highest concentration or were poorly fit and are considered inconclusive. Class 4 curves were inactive, having a curve fit of insufficient efficacy or lacking a fit altogether.

Secondary screen using MLV pseudotyped with MARVGP.

Inhibitors identified from primary screening were tested in a secondary screen at a single concentration of 50 µM for inhibition of MLV pseudotype infection. All treatments and screening was done as duplicates, each replicate in a different plate. Briefly, 2,500 SW-13 cells in 25 µl medium per well were incubated overnight in 384 well tissue culture plates. On the day of assay, test compounds were diluted to 200 µM concentration in complete medium. Twenty five µl of this mixture was added to the cells to achieve a concentration of 100 µM. Twenty five µl of medium was removed from the wells. After 1 h of incubating at 37°C, 25 µl of MLV pseudotype-containing culture supernatant was added to cells. This brought the final concentration of the compound to 50 µM upon addition of virus. All infections were done to achieve a MOI of 0.075 to 0.15. Cells were incubated for 24 h. One day post infection cells were fixed by immersing the plates in formalin for 24 h at 4°C. Formalin from fixed plates was decanted and plates were washed twice with PBS. Nuclei were stained using Hoechst (Life technologies, Cat#H3570) at 1:50,000 dilution and plates were imaged on a Nikon Ti Eclipse automated microscope. Bafilomycin at final a concentration of 10 nM was used as a positive control drug. Nuclei (blue) and infected cells (red) were counted using CellProfiler software.

Testing of compounds for inhibition of infection by wild type MARV and EBOV.

MARV and an infectious EBOV expressing GFP were used in counter screens. Inhibitors identified in secondary screen were tested at 8 concentrations for activity. All treatments and screening was done in duplicates for each virus, each replicate being in a different plate. New lots of compounds from different manufacturer were tested against wild type EBOV. Briefly, 4,000 HeLa cells per well in 25 µl of medium were grown overnight in 384-well tissue culture plates. On the day of assay, test compounds were diluted to 200 µM concentration in complete medium.

25 µl of this mixture was added to the cells already containing 25 µl medium to achieve a concentration of 100 µM. 25 µl of medium was removed from the first wells and added to next well. This type of serial dilution was done 8 times to achieve concentrations of 100, 50, 25, 12.50, 6.25, 3.12, 1.56 and 0.78 µM. One hour after incubating with the compound 25 µl of infection mix containing wild type virus was used to infect cells. This resulted in a final concentration of 50, 25, 12.50, 6.25, 3.12, 1.56, 0.78 and 0.39 µM. Bafilomycin at final a concentration of 10 nM was used as a positive control drug. Primary human macrophages were differentiated from monocytes in 96 well plates. Macrophages differentiated from plating of 50,000 monocytes per well resulted in approximately 3600 macrophages per well. These macrophages were treated with the same concentration of compounds as described above in 100 µl final volume per well.

All wild type virus infections were done in a BSL-4 lab to achieve a MOI of 0.075 to 0.15. Cells were incubated with virus for 24 hours. One day post infection cells were fixed by immersing the plates in formalin overnight at 4°C. Fixed plates were decontaminated and brought out of the BSL-4. Formalin from fixed plates was decanted and plates were washed thrice with PBS. MARV infected plates and wild type EBOV infected plates were immunostained using virus specific antibodies, while plates with GFP encoding EBOV were stained for cell nuclei only. Nuclei were stained using Hoechst at 1:50,000 dilutions. Plates were imaged and nuclei and infected cells were counted using Cell Profiler software (1, 2).

Immunostaining of MARV and EBOV infected plates.

Cells were permeabilized using 0.1% Triton X-100 (Sigma, Cat#T8787) in PBS and blocked for 1 h in 3.5% bovine serum albumin (Fisher-scientific- Cat#BP9704100). Fixed cells were incubated with an anti-MARV VLP antibody (IBT bioservices, Cat#04-0005, 1:1500 dilution) or anti-EBOV GP antibody (IBT bioservices, Cat#0201-020, 1:1500 dilution), overnight at 4°C. After 2 washes

to remove any excess antibody cells were stained with anti-Rabbit Alexa-546 antibody (Life technologies, Cat#A11035). After 3 washes to remove any non-specific antibody nuclei were stained using Hoechst at 1:50,000 dilution and imaged on a Nikon Ti Eclipse automated microscope. Nuclei and infected cells were counted using CellProfiler software.

Effect of compound treatment on transferrin binding.

For binding studies, serum starved and compound treated cells were incubated with 25 µg/ml of transferrin conjugated to Alexa 488 for 20 min. Cells were washed twice with PBS at room temperature to wash off unbound transferrin and fixed in 10% neutral buffered formalin (VWR, Cat#16004-126). Fixed cells were imaged and intensity in 488 channel was measured using cell profiler. Intensity data was exported to FCS express (deNovo software). Non-transferrin treated cell intensities were used to determine baseline and cells corresponding to this intensity were excluded from analysis. Percent of transferrin positive cells for each treatment was plotted.

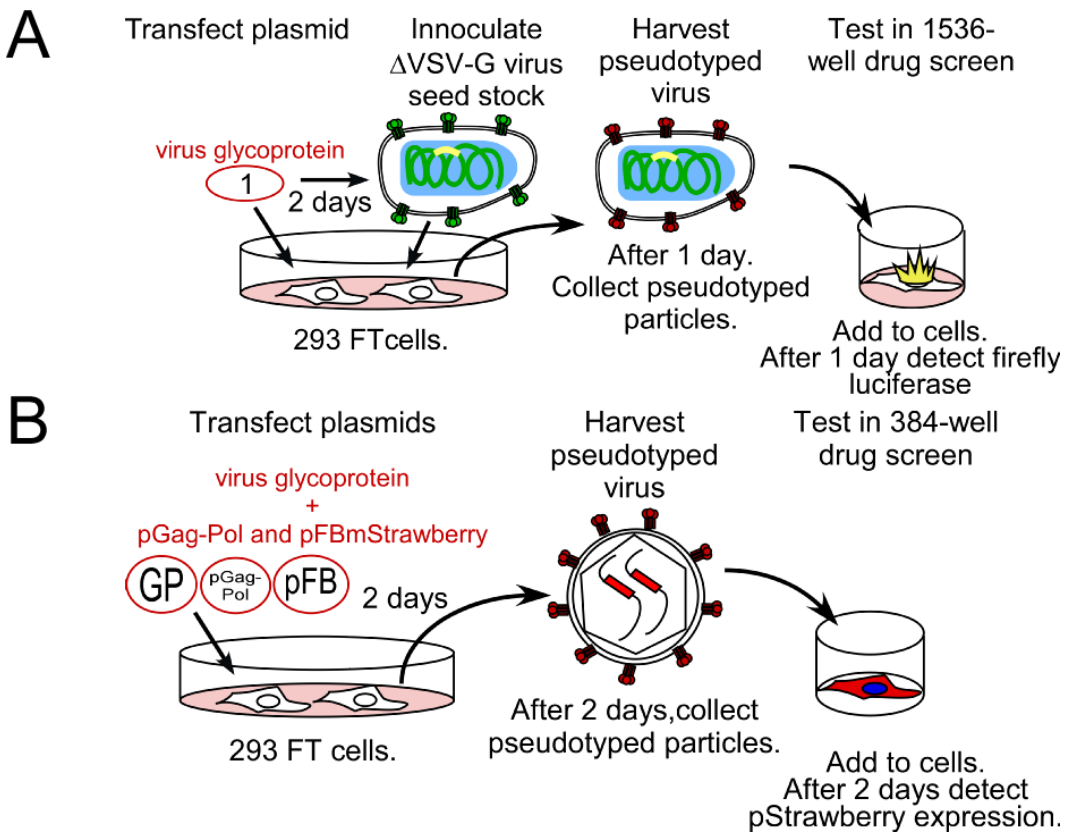

Fig. S1. Pseudotype production process. (a) For preparing Vesicular Stomatitis Virus (VSV) pseudotyped with MARV GP (MARVGP-VSVluc), 293FT cells were transfected with a MARV GP expressing plasmid. 2 d post transfection cells were inoculated with a core VSV stock. This stock virus had a firefly luciferase encoding gene inserted in its genome and expressed Venezuelan Equine Encephalitis (VEE) Virus GP. 1 d post infection culture supernatants containing pseudotyped viruses were harvested and tested for infection by measuring expression of firefly luciferase in infected 293FT cells. (b) For preparing Murine Leukemia Virus (MLV) pseudotyped with MARV GP, 293FT cells were transfected with plasmids expressing MARV GP, gag-pol and viral LTR sequences fused to red fluorescent protein pStrawberry. 2 d post transfection culture supernatants containing pseudotyped viruses were harvested and tested for infection by observing expression of pStrawberry in infected 293FT cells.

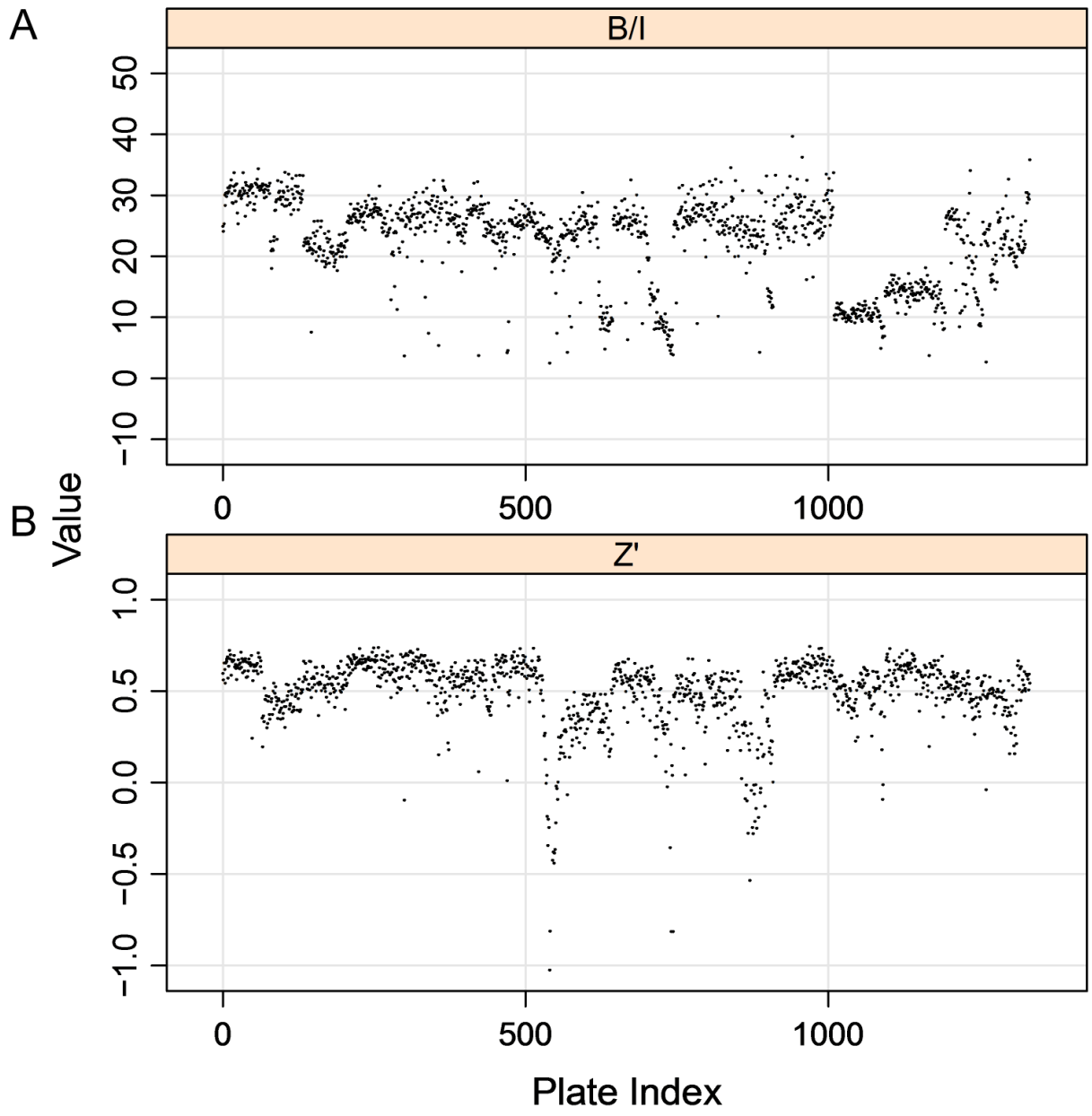

Fig. S2. qHTS screening performance. Each dot corresponds to a 1536-well plate of compounds and controls. (a) Signal to background of assay. (b) Z' trend across entire qHTS. Low Z' plates were rejected and rescreened during course of qHTS.

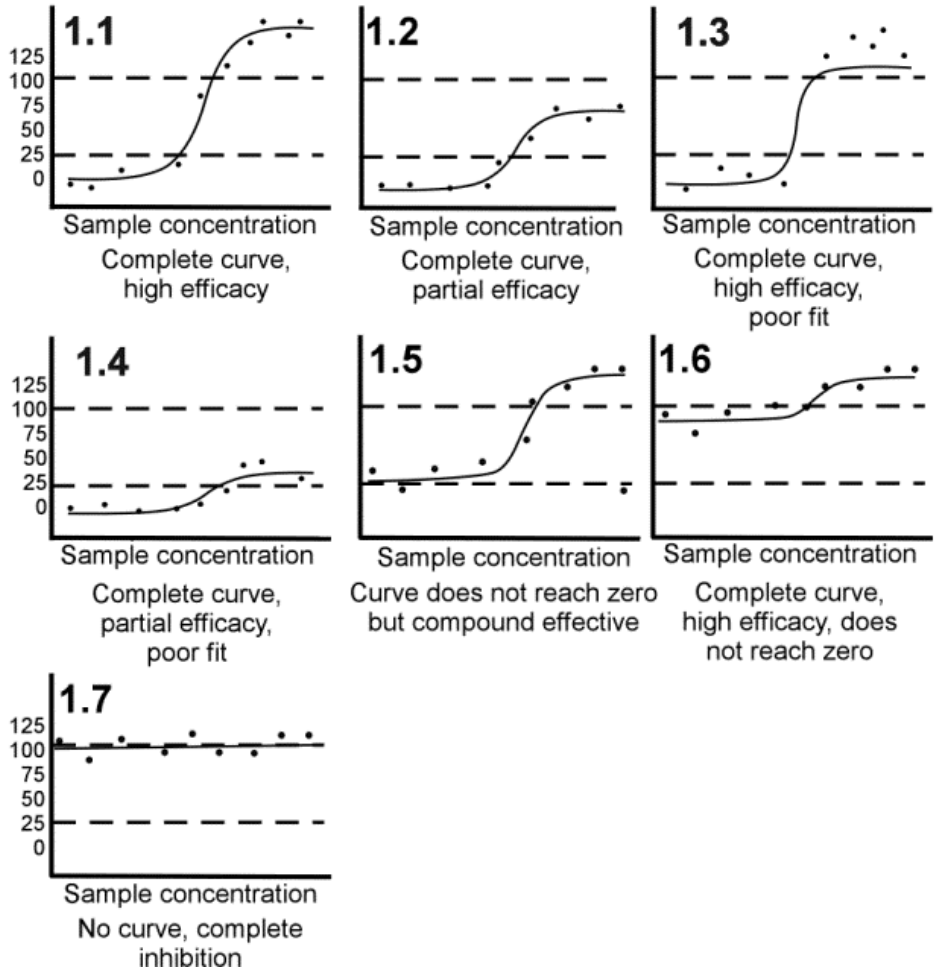

Fig. S3. Curve classification criteria for compounds.1.1: Complete curve; high efficacy: >80% activity; 1.2: Complete curve; partial efficacy:< 90% > 80%; 1.3: Complete curve; high efficacy; poor fit:>80%; 1.4: Complete curve; partial efficacy; poor fit; 1.5: High efficacy incomplete curve as compounds were still found to be active at the lowest dose tested: minimum 25% activity. 1.6: High efficacy incomplete curve as compounds were still found to be active at the lowest dose tested: minimum 75% activity. 1.7: High efficacy no curve as compounds was found to be active at all tested concentrations: 100% activity.

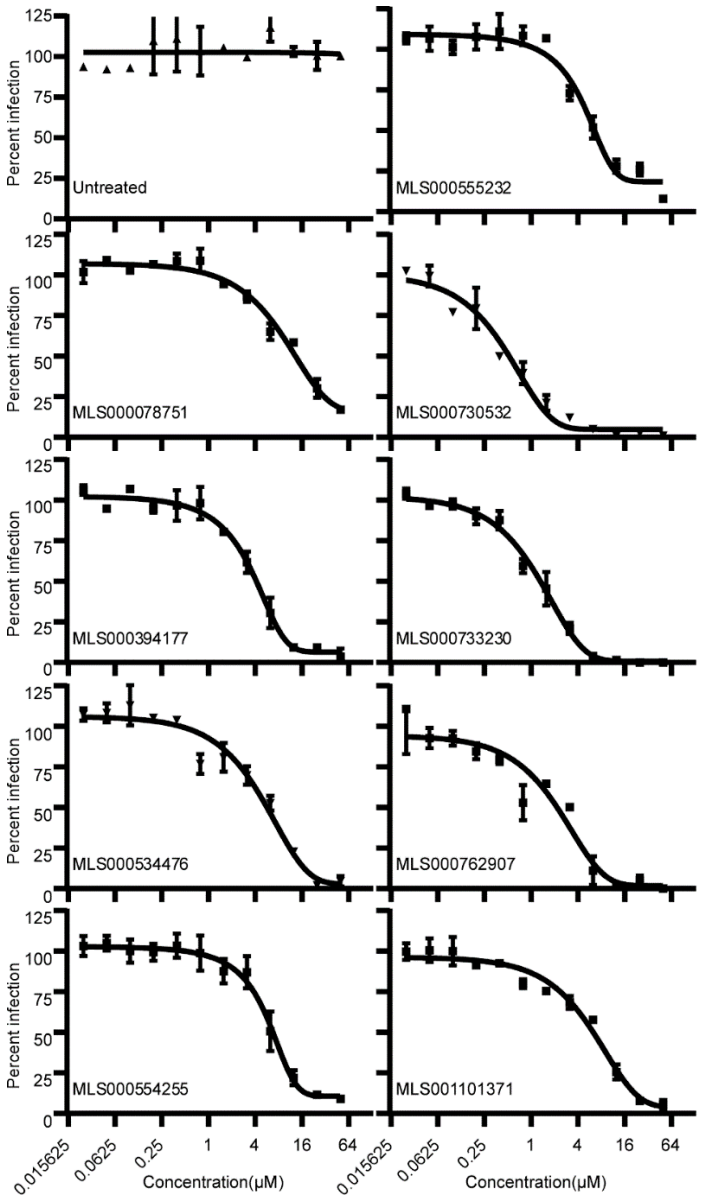

Fig. S4. Dose dependent activity of compounds against MARV in HeLa cells. HeLa cells were pretreated with twelve, half serial dilutions of compounds starting at 50 μM for 1 hour, in duplicates. Treated cells were infected with MARV. One day post infection cells were immunostained using MARV specific antibody. MARV positive and total cell numbers were counted. A ratio of infected cell number to total cell number was used to determine percent infection.

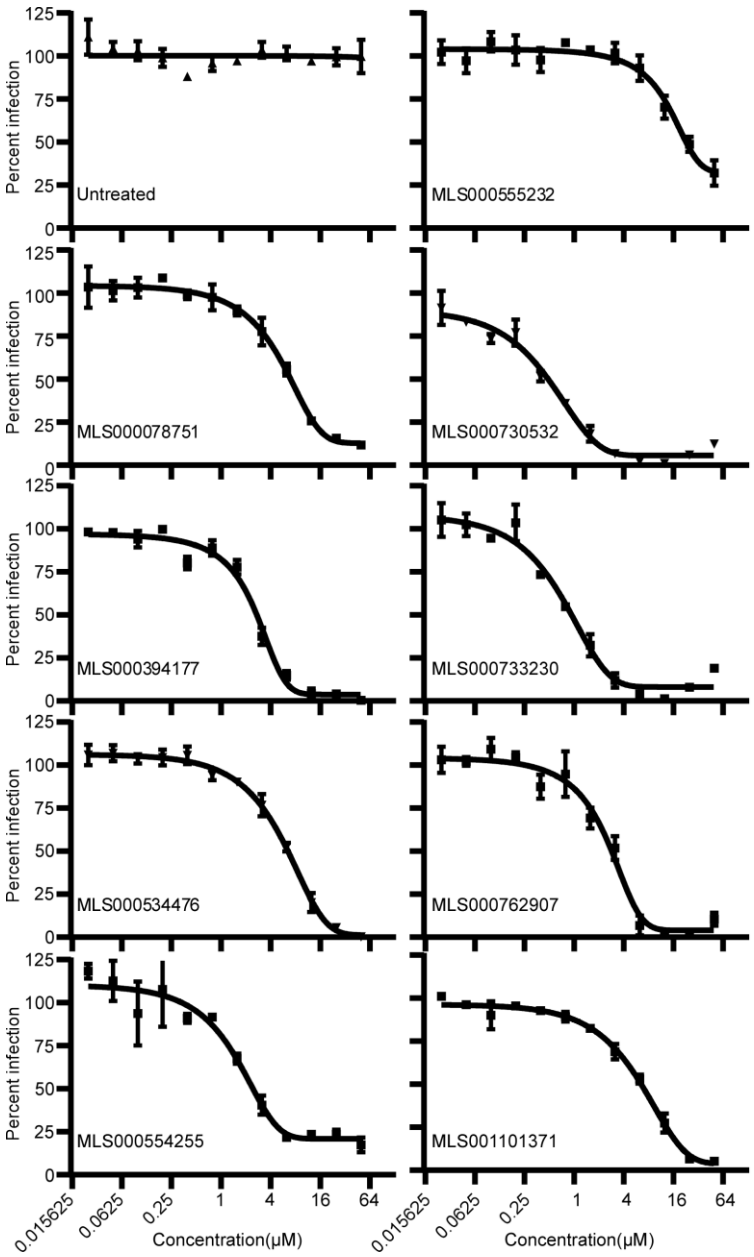

Fig. S5. Dose dependent activity of compounds against EBOV-GFP in HeLa cells. HeLa cells were pretreated with twelve, half serial dilutions of compounds starting at 50 μM for 1 hour, in duplicates. Treated cells were infected with EBOV-GFP. GFP positive cells and total cell numbers were counted, 1 d after infection. A ratio of infected cell number to total cell number was used to determine percent infection.

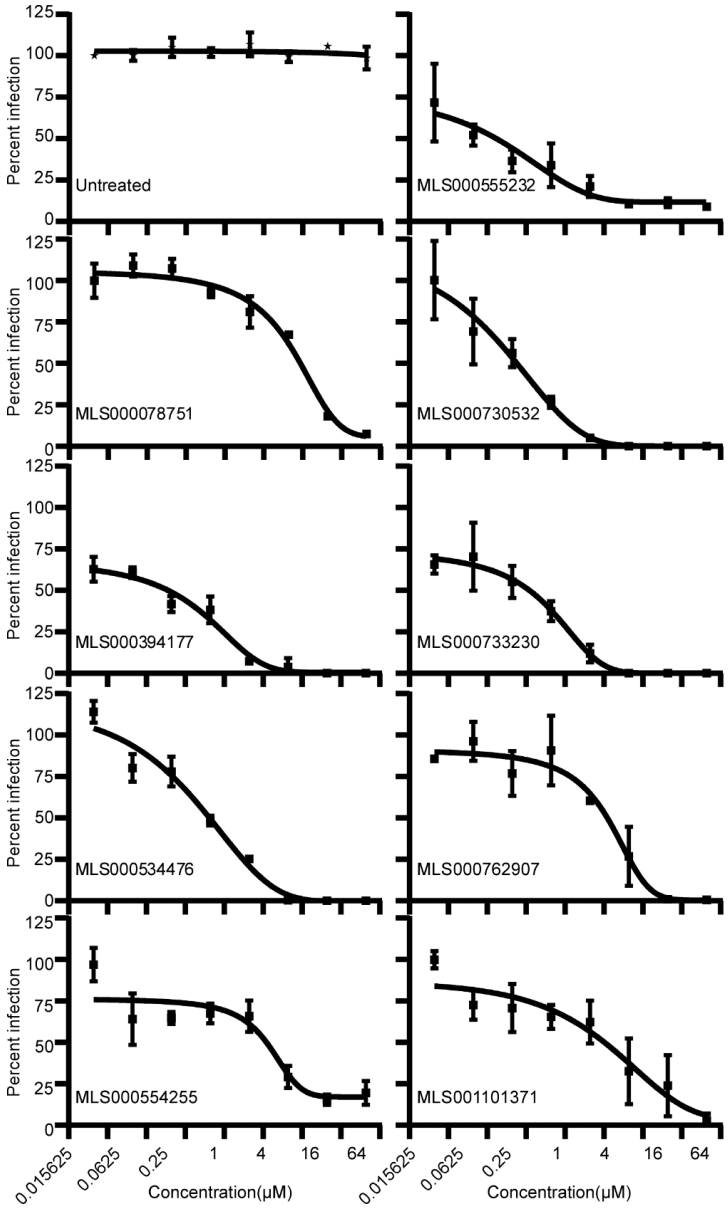

Fig. S6. Dose dependent activity of compounds against EBOV in HeLa cells. HeLa cells were pretreated with twelve, half serial dilutions of compounds starting at 50 μM for 1 hour, in duplicates. Treated cells were infected with EBOV. One day post infection cells were immunostained using EBOV specific antibody. EBOV positive and total cell numbers were counted. A ratio of infected cell number to total cell number was used to determine percent infection.

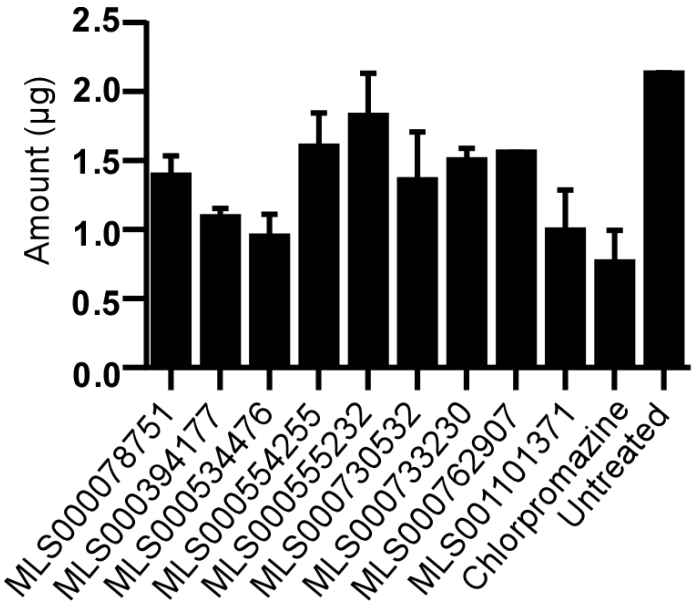

Fig. S7. Effect of compound treatment on binding of transferrin to cells. HeLa cells were serum starved for 4 hours followed by treatment with compounds in triplicates for 1 hour in serum free medium. Treated cells were then transferred to 14°C. Cells were incubated with 25 µg/ml of human transferrin for 40 minutes followed by washing and lysis. Transferrin bound to cells was estimated using ELISA. Bafilomycin A (10 nM) an inhibitor of endosomal acidification, EIPA (25 µM) a specific inhibitor of macropinocytosis and Chlorpromazine (25 µM) an inhibitor of clathrin mediated endocytosis and known to block transferrin uptake were used as controls. All assays were performed 3 times with similar outcomes.

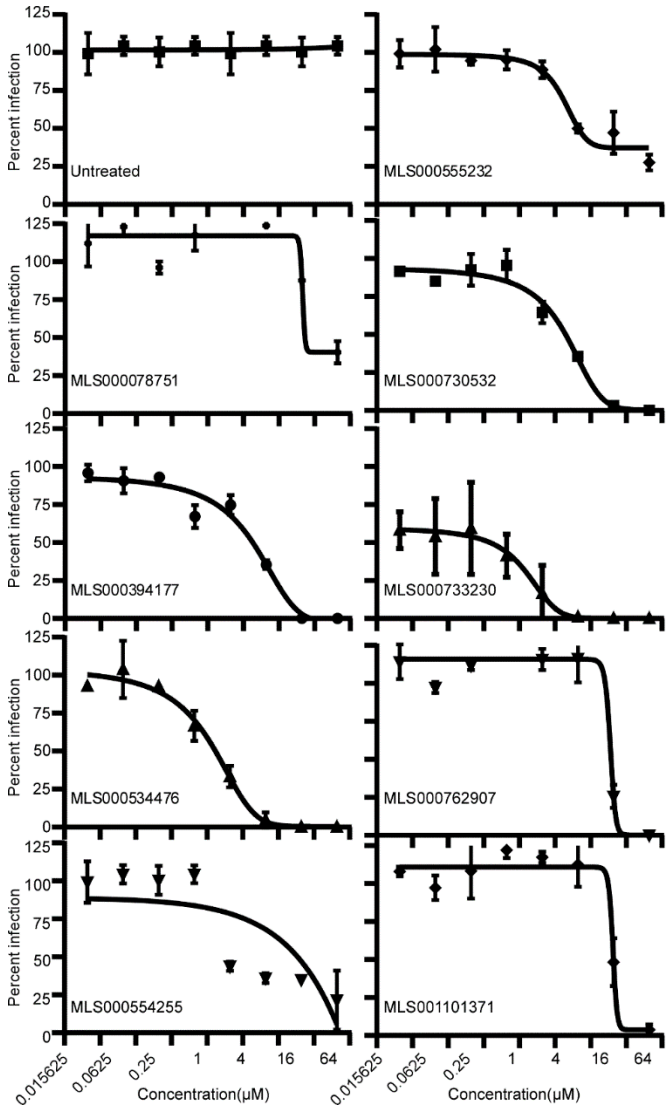

Fig. S8. Dose dependent activity of compounds against EBOV in primary human macrophages. Human macrophages were differentiated from monocytes derived from human blood. Cells were pretreated with twelve, half serial dilutions of compounds starting at 50 μM for 1 hour, in duplicates. Treated cells were infected with EBOV. One day post infection cells were immunostained using EBOV specific antibody. EBOV positive and total cell numbers were counted. A ratio of infected cell number to total cell number was used to determine percent infection.

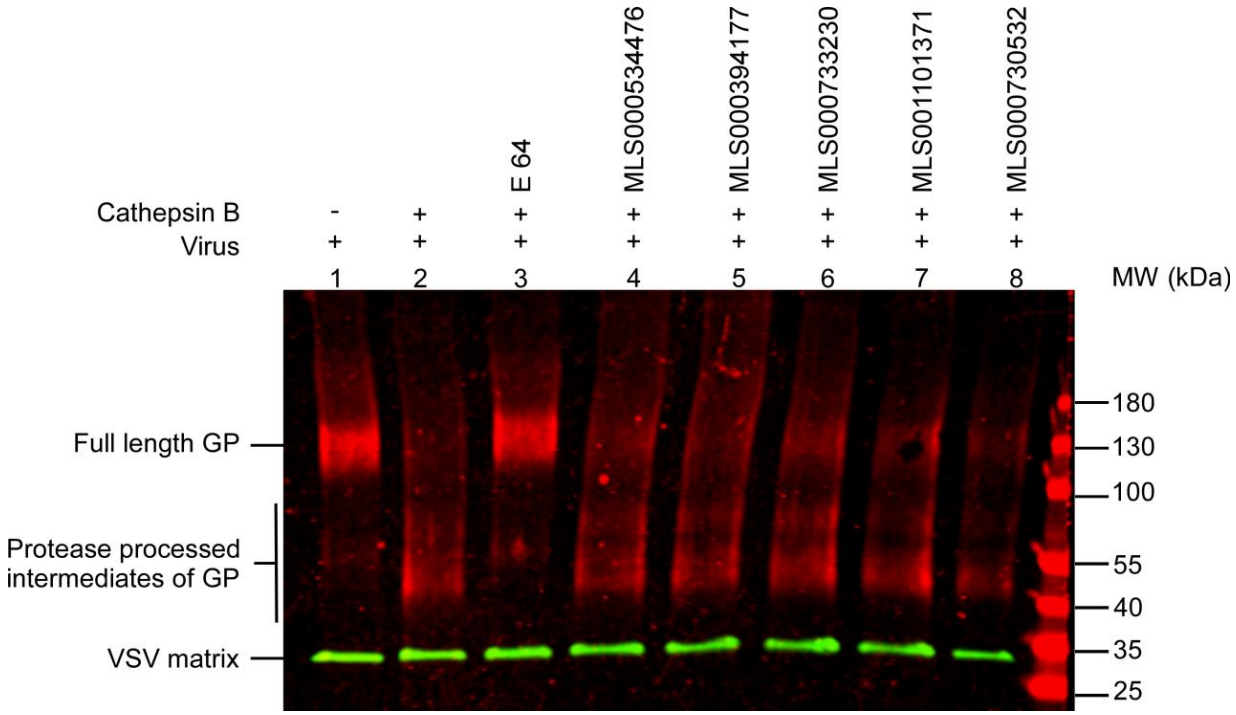

Fig. S9. Representative example of cathepsin B inhibition studies. EBOV GP/VSV pseudovirions were incubated with the indicated compounds (50 µM) and cathepsin B for 15 min at 37°C. Proteins in samples were separated on a SDS PAGE, transferred to nitrocellulose and immunoblotted for EBOV GP (red) and VSV matrix (green). Within each lane, the remaining amount of full length EBOV GP was calculated based on average pixel intensity and normalized to the signal from VSV matrix as a loading control. All studies included the following controls: untreated virus (lane 1), cathepsin B treated virus (lane 2) and cathepsin B treated virus in the presence of the cathepsin inhibitor E64 (30 µM).

Table S1. Potency of compounds against wild type MARV and EBOV. Compound efficacy was classified based on completeness of the dose response curve (Curve class as defined in Supplementary Fig. 3) and concentration of each resulting in 50% infection inhibition (IC-50).

Compound name	**SMILES**	**Curve class MARV**	**IC-50 MARV (µM)**	**Curve Class EBOV**	**IC-50 EBOV (µM)**
MLS0000787 51	OC1=CC=C (C=C1)C (=C)C2=CC=C (O)C=C2	1.5	6.128	1.3	25.6
MLS0003941 77	C1=CC=C (C=C1)N2C (=NN=N2)SC3=CC= C (C=C3[N+] (=O)[O-])S (=O) (=O)NCCN4CCOCC4	1.3	12.89	1.6	1.888
MLS0005344 76	NCCNC1=NC (C2=CC=CC=C2)=C3 CCCCC3=C1C#N	1.3	6.211	1.6	24.4
MLS0012393 25	Cl.CN (C)CCCN (C (=O)C1=C/C=C2/C=C \C=C/C\2=C\1)C3=N/	1.3	13.28	1.7	1.554

	C4=C/C5=C (OCO5)\C=C/4S\3				
MLS000555232	COC1=CC2=C (C=C1)N=C3C=C (Cl)C=CC3=C2NC4=CC (CN5CCN (C)CC5)=C (O)C=C4	1.3	6.258	1.6	1.744
MLS000730532	CC=1C=C (N=C2C1C=C (C=C2)NC (=NCCCN3CCC4=CC=CC=C4C3)S)N5CCN (CC5)C	1.3	1.929	1.1	1.557
MLS000733230	CC1=CC (=C (S1)C)C=2C (=CN (N2)C3=CC=CC=C3F)CN4CCN (CC4)C5=CC=NC=C5	1.3	11.67	1.3	6.698
MLS000762907	CC=1C (=C2N=C3C (=C (N2N1)NCCCN (C)C)CCCC3)C4=CC=CC=C4	1.3	12.54	1.3	3.188

MLS001101371	CC (C)N1CCN (CC1)C (=O)C2=C/C=C (OC3CCN (CCC4=C/C=C\C=C\4)CC3)\C=C\2	1.3	12.96	1.3	12.92
MLS001030162	CC1=C (C2=CC (=CC=C2N1CC3=CC =CC=C3)OC)CC[NH 3+].[Cl-]	1.1	25.32	1.3	6.405
MLS000666802	CC (C) (C)C1=CC (=CC=C1OCCOCCN CC=C)Cl.C (=O) (C (=O)O)O	1.1	25.17	1.5	0.6157
MLS000999960	Cl.CC1=C/C (NC (=O)CCN2CCN (C\C=C\C3=C\C=C/C =C\3)CC2)=C\C=C\1 Br	1.1	26.4	1.6	3.276
MLS001173370	Cl.FC1=C/C=C (\C=C\1)C (=O)CCCN2CC\C3=C	1.1	25.14	1.3	3.058

	(C2)\C4=C\C (F)=C/C=C\4N3				
MLS001174896	O=C (C#CC1=C/C=C\C=C\1)C2=C/C=C\O\2	1.1	12.25	1.1	12.38
MLS001196083	Cl.CC1=C/C (Cl)=C\C (C)=C\1OCCN2\C=C/N=C\2	1.1	2.058	1.1	6.187
MLS001239358	Cl.CCOC (=O)N1CCN (CC1)S (=O) (=O)C2=CC=C (C=C2)C (=O)N (CCCN (C)C)C3=NC4=C (C)C (C)=CC=C4S3	1.1	13.04	1.6	0.6092
MLS001177927	Cl.CC (NCC (O)COC1=C (C)/C=C\C=C\1C)C2=C/C=C\C=C\2	1.2	25.77	1.5	6.424
MLS001209453	COC1=C/C=C (NC2=C/C	1.2	12.47	1.3	3.403

	(C)=N\C3=C\C=C (OC)/C=C\2\3)\C=C\1				
MLS000580080	CCOC1=CC=CC (=C1OCC2=CC=C (C=C2Cl)Cl)CNCCO.Cl	1.2	25.05	1.3	24.6
MLS000106706	CC1=CC (OCCCCCNCCO)=C C (C)=C1Cl	1.2	25.24	1.5	0.5953
MLS001175455	CCN (CC)CCNC (=O)C1=C/C=C (NC (=O)C2=C/C=C\C=C\2\C3=C\C=C (/C=C\3)C (F) (F)F)\C=C\1	1.2	25.45	1.6	6.419
MLS000772389	CCN (CC)CCCNC=1C=2C 3=C (SC2N=C (N1)CN4CCOCC4)C CC3	1.2	24.76	1.6	3.508
MLS000042810	CC1CC (=C)CC (O1)C2=CC=CC=C2	1.2	25.5	1.7	~ -2.829

	OCC (O)CN3CCCCC3				
MLS001162808	COC1=C/C=C\C=C\1 CCN2CCCC (CN (C)CC (C) (C)CO)C2	1.2	26.09	1.3	7.248
MLS000684231	CCOC (=O)C1=C (N (C=2C1=CC (=C (C2)Br)OC)CC (CN (C)C)O)C.Cl	1.2	4.878	1.3	6.477
MLS000556387	CCN (CC)CCNC1=NCCN2 C1=CC3=C2C=CC (OC)=C3	1.2	25.46	1.6	2.929
MLS001179278	COC1=CC2=C (SC3=C (C=C (OC)C=C3)C (C2)N4CCN (C)CC4)C=C1	1.2	12.5	1.6	6.49
MLS000567464	CC1=CC (=C (S1)NC (=O)CCl)C (=O)C2=CC=CC=C2	1.2	~ 44.36	1.6	25.21
MLS000583959	CC1=CC=C (C=C1)N (C (C2=CC=C	1.2	12.13	1.3	12.4

	(C=C2)OC)C (=O)NC3CCCCC3)C (=O)CCl				
MLS001018888	CCN1CCCC1CNCC (COC2=CC=C (C=C2C)C (C) (C)C)O.C (=O) (C (=O)O)O	1.2	25.65	1.3	12.23
MLS001047402	CN (C)C1=CC (=C2C=CC=CC2=N1) NC (=O)CN3CCCC3.C (=O) (C (=O)O)O	1.2	25.93	1.2	5.259
MLS001029946	CC1=CC (=NC (=N1)N2CCOCC2)N C3=CC=C (C=C3)Cl	1.2	~ 43.21	1.6	1.522
MLS000332573	CC1 (CC (CC (N1) (C)C)NC (=O)N2C3=CC=CC= C3SC=4C2=CC=CC4)C	1.2	12.76	1.3	6.022
MLS000936259	COC1=C/C=C (NC (=O)C2C3OC4	1.2	25.34	1.3	12.46

	(\C=C/3)C2C (=O)N (CCN5CCCCC5)C4C (=O)NC6CCCC (C)C6C)\C=C\1				
MLS000718690	CCOC1=CC (=CC (=C1OC)Cl)CNCCC2 =CNC=3C2=CC=CC3 .Cl	1.2	25.33	2.1	2.77
MLS000880330	FC1=CC=C (C=C1)C (N2CCN (CC2)C3CCCCC3)C4 =NN=NN4CC5=CC= CS5	1.2	25.77	1.6	6.385
MLS001207131	CN (C)CCCN=C (S)N1CCN (CC1)C2=N/C (=C\S\2)/C3=C/C=C\ C=C/3	1.2	25.29	1.3	25.21
MLS000673118	CCCN1CCN (CC1)CCCNC (=O)C2=CC=3C (S2)=C4C=C (C=CC4=NC3O)C	1.2	25.11	1.3	25.01

MLS000689481	CCCCN (CCCC)CCNC (=O)CSCC1=CC=C (C=C1)C.C (=O) (C (=O)O)O	1.2	12.81	1.3	12.03
MLS000554255	CC (C) (CS ([O-]) (=O)=O)NC (=O)CC[N+] (C) (C)CCO	1.3	6.393	1.5	6.843
MLS001179272	FC1=CC2=C (C=C1)C (CC3=C (S2)C=CC (Cl)=C3)N4CCN (CCC5OCCO5)CC4	1.3	25.41	1.5	0.6907
MLS002702500	COC1=C (OC)C=C (C=C1)C2C (COC (C)=O)C (=C)C (=O)C3=C2C=CS3	1.3	2.061	1.1	5.931
MLS001180408	CN (C)CCCNC (=O)C (\NC (=O)C1=CC=C (C)C=C1)=C/C2=CC=	1.1	12.48	4	

	C (O2)C3=CC=C (C=C3)[N+] ([O-])=O				
MLS000737975	OC1=C/C=C (NC2=C/C=N\C3=C\C (Cl)=C/C=C\2\3)\C=C\1	1.1	25.23	4	
MLS001237534	CC1=C/C=C\C=C\1NC (=O)CSC2=C/C=C (\N=N\2)/C3=C/C=C\C=N/3	1.1	24.81	4	
MLS000336293	CC1=CC=C (C=C1)NC (=O)CN (C)C (=O)CN2C (=O)C (=C (C=N2)Cl)Cl	1.1	24.03	4	
MLS000780111	C=1C=C2C (=CC1C (=O)CCl)NC (=O)CO2	1.1	~ 38.84	4	
MLS001179997	OC (=O)C (O)=O.CCN (CCNCC (O)COC1=C/C=C\C=C\1C	1.1	25.5	1.4	

	(C)C)C2=C/C=C\C (C)=C\2				
MLS000418057	C1=CC=C (C=C1)CN2CCC (CC2)NC (=S)NC3=CC=C (C=C3)Cl	1.2	25.6	4	
MLS000936795	CN (C)CCNC (=O)C1=C (C)/C2=C/C (=C\C=C/2O\1)S (=O) (=O)N3CCCC3	1.2	25.38	1.4	
MLS000092187	ClC1=CC=C (C=C1)N2C (N3CCCC3C2=O)C4 =CC=CS4	1.2	25.19	1.4	
MLS000391391	C1=CC=C (C=C1)C2=CSC (=N2)NC (=O)COC (=O)/C=C/C3=CC=C S3	1.2	25.29	1.4	

MLS000569139	CC1=CC=C(C=C1)NC(=O)ON=C2CCCC2	1.2	12.7	1.4	
MLS001045935	COC1=CC=CC=C1C(=O)ONC(=N)C2=CC=CC=N2	1.2	27.89	1.4	
MLS001181479	OC (CN1C2=C/C=C (Cl)\C=C\2\C3=C\C (Cl)=C/C=C1\3)CN4CCCCC4	1.2	25.62	4	
MLS000391984	CC (C) (C)C1=CC (=CC (=C1O)C (C) (C)C)C=NO	1.2	6.121	1.4	
MLS000063146	O=C (NC1CCCCC1)C2=CC=C (C=C2)S (=O) (=O)N3CCCCC3	1.2	25.39	4	
MLS000768081	CC1=CC (=C2C (=C1)C (=CC (=N2)C)NC3=CC=C (C=C3)C (=O)NCCO)C	1.2	13.83	4	

MLS000091920	CCN1CCN (CCCNC (=O)C2=CC3=CC4=CC=C (OC)C=C4N=C3O2)CC1	1.2	5.397	4	
MLS001150589	COC1=CC=C (C=C1)C (CNC (=O)C2=C (C)C3=CC (OC)=CC=C3O2)N4CCCC4	1.2	25.87	4	
MLS000736237	C=1C=C (C=CC1CN2CCN (CC2)C3=CC=C (C=C3)O)OCCCN4CCCCC4	1.2	13.99	4	

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