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Electronic Supplementary Material

Screening and Identification of Marburg Virus Entry Inhibitors using Approved Drugs

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Supplementary Materials and Methods

Cell lines, pseudoviruses and compound library

293T cells (ATCC, CRL-3216) were cultured in a complete Dulbecco's modified Eagle's medium (DMEM, Hyclone, SH30243.01B) supplemented with 10% fetal bovine serum (Gibco, 106000644), 1% penicillin–streptomycin solution (GIBCO, 15140163) and 2% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Gibco, 15630080). Cells were grown in a 5% CO_2 environment at 37 °C.

The HIV-based MARV pseudovirus (p/HIV/MVGP/Fluc) expressing MARV GP and firefly luciferase reporter protein (Fluc) was generated by co-transfection of 293T cells with pCDNA3.1–MARV-GP and envelope-defective HIV-1 SG3, as described previously (Zhang *et al*., 2017). The VSV pseudovirus p/HIV/VSVG/Fluc was produced as previously described (Nie *et al*., 2017).

A total of 767 standard compounds were obtained from the National Standard Chemical Control Library of the National Institutes for Food and Drug Control, China (NIFDC). The purity of each compound was >95%, as verified by high performance liquid chromatography analysis. All compounds are clinically approved drugs and were selected based on their high pharmacological diversity as well as their guaranteed bioavailability and safety

in humans.

The compounds were dissolved at 30 mmol/L in dimethyl sulfoxide (DMSO), aliquoted and stored at –20°C.

In vitro anti-MARV screening

The anti-MARV activity screening of the compound library was performed in 96-well plates. Compound with a final concentration of 200 μmol/L was preincubated with p/HIV/MVGP/Fluc (200 $TCID_{50}$/well) in 100 L complete DMEM at 37°C, 5% CO_2 for 1 h. After incubation, 5×10^4 293T cells were seeded to each well in 100 L complete DMEM. Cell controls and viral controls were arranged in each plate on columns 1 and 2. After 48 h incubation at 37°C with 5% CO_2, culture medium was aspirated gently to leave 100 μL in each well. Bright-Glo luciferase reagent (100 μL; Promega, Madison, WI) was added to each well, and luminescence was measured as previously described using a Glomax 96 microplate luminometer (Promega, Fitchburg, WI) (Zhang et al., 2017). The percent inhibition was calculated using the following equation: 100× [1- (RLU in the presence of compound - RLU of cells control) / (RLU of virus control - RLU of cells control)].

Compounds with an inhibition rate of over 70% were selected for hit confirmation and serially diluted (4 or 8 dilutions in total) for IC_{50} calculation. Serial dilutions were performed in duplicate with an initial concentration of 200 μmol/L.

Cell viability assay

Cytotoxicity testing was performed to determine the 50% cytotoxic concentration (CC_{50}) of the test compound in the absence of pseudovirus. Compounds were serially diluted in 96-well-plates. 293T cells were seeded at 5×10^4 cells/well, and incubated at 37°C, 5% CO_2 for 48 h. The cytotoxicity was examined using the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

In vivo anti-MARV bioluminescence imaging (BLI) analysis

Four to five-week old female Balb/c mice were obtained from the Institute for Laboratory Animal Resources of NIFDC, and the study protocol was approved by the Animal Care and Use Committee of NIFDC. To challenge mice, 2.6×10^6 $TCID_{50p/HIV/MVGP/Fluc}$ was delivered by intraperitoneal (IP) injection to groups of 3 mice per drug.

The dose, delivery route and timeline of the drug administration are described in Supplementary Table S1. Bioluminescence imaging (BLI) analysis was performed with the IVIS Lumina Series III Imaging System (PerkinElmer, Baltimore, MD) as previously described (Zhang et al., 2017). Living Image software (Caliper Life Sciences, Baltimore, MD) was used to analyze the regions of interest, which were presented as the total flux in photons/s.

Data analysis and statistics

The Z′factor was calculated from the normalized signals of positive control (DMSO, 100% response) and negative control (293T cells, 0% response) using the following equation: $1 – 3(Std_{DMSO}+ Std_{293T})/(Mean_{DMSO}– Mean_{293T})$. The coefficient of variation (%) was calculated with the following equation: 100× Std duplicate wells/Mean duplicate wells. The inhibition rate of the compound, IC_{50} and CC_{50} values and bioluminescence imaging data were analyzed and calculated using excel and GraphPad Prism 6.0 software (GraphPad, San Diego, CA).

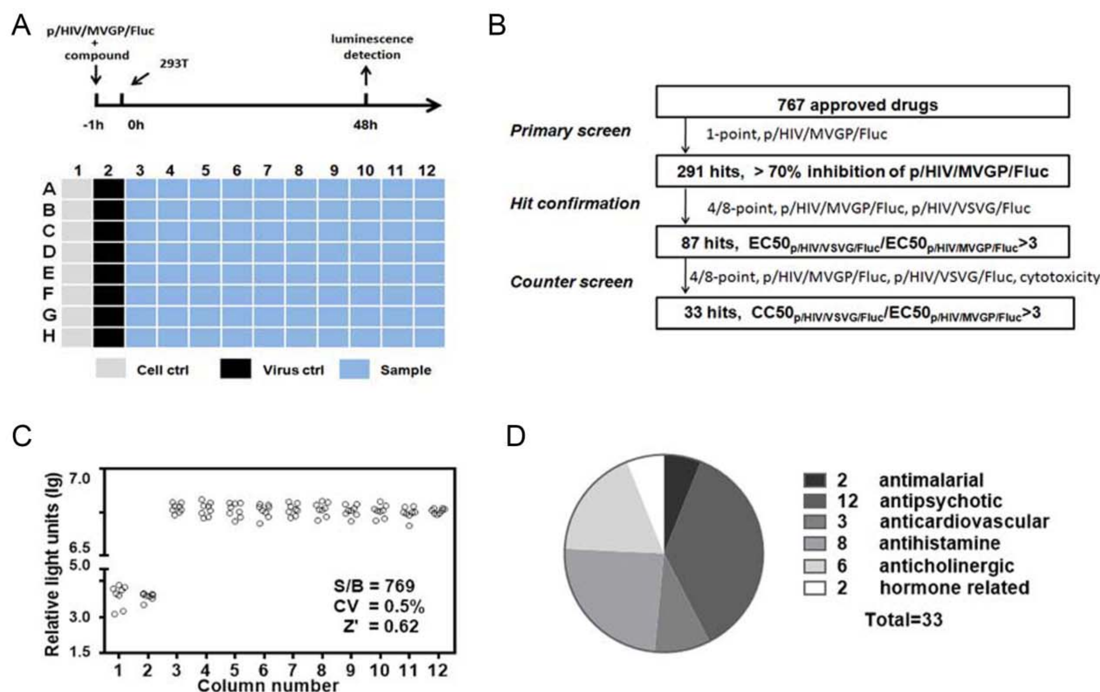

Fig. S1. *In vitro* screening of anti-MARV compounds.

(A) The schematic diagram of the protocol and plate design for the MARV pseudovirus screening system *in vitro*. Wells in column 1 represented the cell control and contained only 293T cells. Wells in column 2 represented the virus control and contained 293T cells infected with p/HIV/MVGP/Fluc. Wells in columns 3 to 12 represented the experimental samples and contained 293T cells infected with p/HIV/MVGP/Fluc and treated with varying concentrations of each compound. All concentrations for all compounds were tested in duplicate.

(B) Scatter plot of the results from a DMSO plate screening. Wells in columns 1 and 2 of the 96-well assay plates contained 293T cells as a control (negative control, 0% response). p/HIV/MVGP/Fluc (positive control, 100% response) was added to all other wells along with 0.66% DMSO, in which most of the compounds were diluted. The signal-to-noise (S/B) ratio, coefficient of variation (CV) and Z' factor are indicated on the graph.

(C) Flowchart of the serial screenings. In the primary screen, the inhibitory rate of 767 compounds against p/HIV/MVGP/Fluc was examined at a concentration of 200 μmol/L (1-point). In the confirmation screen, the positive hits from the first screen were tested against both p/HIV/MVGP/Fluc and p/HIV/VSVG/Fluc at concentrations starting from 200 μmol/L (1:3 serial dilutions, 4/8-points). Finally, in the counter screen, the anti-MARV effect of the positive hits (defined as having an $EC_{50p/HIV/VSVG/Fluc}$ / $EC_{50p/HIV/MVGP/Fluc} > 3$) was investigated at varying concentrations (1:3 serial dilutions, 4/8-points), against both p/HIV/MVGP/Fluc and p/HIV/VSVG/Fluc. A cytotoxicity screen was performed in parallel, and positive hits were defined as having $CC_{50p/HIV/VSVG/Fluc}$ /$EC_{50p/HIV/MVGP/Fluc} > 3$.

(D) The distribution of the candidate anti-MARV drugs according to their individual approved indications.

Table S1. Drug administration methods for *in vivo* anti-MARV BLI analysis

Compounds	Solvent	Dose (mg/kg)	Route	Timeline
Chloroquine phosphate	S1	75	IP	A
Amodiaquine hydrochloride	S1	90	IG	A
Mianserin hydrochloride	S1	5	IP	B
Diltiazem hydrochloride	S1	50	IP	B
Imipramine hydrochloride	S1	45	IP	C
Pizotyline	S3	4.5	IP	C
Sertraline hydrochloride	S3	20	IP	C
Promethazine hydrochloride	S1	11.25	IP	C
Budesonide	S2	0.25	IP	C
Tolterodine tartrate	S4	0.6	IP	C
Propafenone hydrochloride	S2	135	IP	C
Defluoroxy paroxetine	S3	3	IP	C
Azelastine hydrochloride	S2	0.6	IP	C
Trihexyphenidyl hydrochloride	S1	1.5	IP	C
Propiverine hydrochloride	S1	3	IP	C
Phencynonate hydrochloride	S2	0.6	IP	C
Amitriptyline hydrochloride	S1	30	IP	C
Flutamide	S2	112.5	IP	C
Benproperine phosphate	S1	18	IP	C
Cloperastine hydrochloride	S1	9	IP	C
Dicyclomine hydrochloride	S1	12	IP	C
Haloperidol	S2	13.5	IP	C
Oxybutynin chloride	S1	3	IP	C
N- methylparoxetine	S4	45	IG	C
Ketotifen fumarate	S4	0.3	IP	C
Terfenadine	S4	18	IP	C
Clemastine fumarate	S3	0.4	IP	C
Fluphenazine	S2	3.75	IP	C
Fluoxertine hydrochloride;	S2	6	IP	C
Paroxetine hydrochloride	S1	6	IP	C
Astemizole	S2	1.5	IP	C
Flupenthixol hydrochloride	S4	2.7	IP	C
Amlodipine besylate	S4	1.5	IP	C

S1: Sterile water for injection; S2: 0.5% methylcellulose (with 1.6% Ethanol); S3: 0.5% methylcellulose (with 1.6% DMSO); S4: 0.5% methylcellulose. A: drug was injected at -2 days post injection (-2dpi), -1dpi and -4 hours post injection (-4hpi). B: drug was injected at -1dpi and 4 hpi, 1dpi and 2dpi. C: drug was injected at 4hpi and 1dpi.
IP: intraperitoneal injection; IG: intragastric injection

References

Nie J, Wu X, Ma J, Cao S, Huang W, Liu Q, *et al*.(2017) Development of in vitro and *in vivo* rabies virus neutralization assays based on a high-titer pseudovirus system. Scientific reports; 7: 42769