Project Title

Host target therapeutics as a broad-spectrum antiviral for medically important positive-sense RNA viruses.

Project Lead and Members

Project lead: Associate Professor Justin, Jang Hann CHU

Project members: Kan Xing Wu, Thinesshwary Yogarajah, Marcus Wing Choy Loe, Parveen Kaur, Regina Ching Hua Lee, Chee Keng Mok, Yi Hao Wong, Patchara Phuektes, Li Sze Yeo, Vincent T.K. Chow, Yong Wah Tan

Organisation(s) Involved

1. Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, National University Health System, National University of Singapore, MD4 Level 5, 5 Science Drive 2, Singapore 117597, Singapore.

2 Infectious Disease Programme, Yong Loo Lin School of Medicine, National University of Singapore 117597, Singapore

3 Institute of Molecular and Cell Biology, Agency for Science, Technology and Research (A*STAR), Singapore

4 NUSMed Biosafety Level 3 Core Facility, Yong Loo Lin School of Medicine, National University of Singapore, 14 Medical Drive, Singapore 117599

5 Department of Pathobiology, Faculty of Veterinary Medicine, Khonkaen University, Khonkaen, 40002, Thailand

Healthcare Family Group(s) Involved in this Project

Medical

Applicable Specialty or Discipline

Infectious Disease, Host-Viral interaction, Antivirals

Project Period

Start date: 1st January 2015

Completed date: 31st October 2022

Aims

1. Our study defines that plant derived saponin (peruvoside) metabolite triggers the pleotropic cellular pathway of Src kinase signalling downstream regulation of the novel CDK1 signalling that phosphorylates GBF1 and in-turn causing Golgi vesiculation which disrupts viral replication.

2. Our study identifies peruvoside as broad-spectrum antiviral against 4 families of viruses and demonstrates antiviral activity against SARS-CoV-2 with minimal in vitro and in vivo cytotoxicity.

3. Our findings pave the way for consideration of host-directed antivirals for current virus-mediated disease, in the interim where no vaccine is available. These findings would be of considerable interest to scientists, clinicians, public health officials, and policy makers globally.

Lessons Learnt

Positive-sense RNA viruses modify intracellular calcium stores, endoplasmic reticulum and Golgi apparatus (Golgi) to generate membranous replication organelles known as viral factories. Viral factories provide a conducive and substantial enclave for essential virus replication via concentrating necessary cellular factors and viral proteins in proximity. Here, we identified the vital role of a broad-spectrum antiviral, peruvoside in limiting the formation of viral factories. Mechanistically, we revealed the pleiotropic cellular effect of Src and PLC kinase signaling via cyclin-dependent kinase 1 signaling leads to Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1) phosphorylation and Golgi vesiculation by peruvoside treatment. The ramification of GBF1 phosphorylation fosters GBF1 deprivation consequentially activating downstream antiviral signaling by dampening viral factories formation. These findings highlight the importance of dissecting the broad-spectrum antiviral therapeutics mechanism and pave the way for consideration of peruvoside, host-directed antivirals for positive-sense RNA virus-mediated disease, in the interim where no vaccine is available.

Additional Information

Singapore Health & Biomedical Congress (SHBC) 2022: Singapore Young Investigator Award (Basic Science & Translational Research) (Oral category) – (Bronze Award); Our current approach to understanding the role of Peruvoside in positive-strand RNA virus replication inhibition lies in the conjecture of a direct role in viral replication inhibition via the viral replication factory formation, it was a challenge to look at various RNA viruses that use different cells lines, different pathways for infection. It is crucial to look for similarities between these virus infections and replication to identify a broad-spectrum inhibitor.

Project Category

Applied/ Translational Research

Quantitative Research

Keywords

Research and Development, Antivirals, Infectious Diseases, RNA, GBF1, phosphorylates

Name and Email of Project Contact Person(s)

Name: Dr. Thinesshwary Yogarajah

Email: micthiy@nus.edu.sg

Host target therapeutics as a broad-spectrum antiviral for medically important positive-sense RNA viruses.

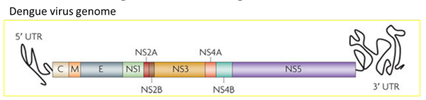
Kan Xing Wu*[1], **Thinesshwary Yogarajah***[2], Marcus Wing Choy Loe[2], Parveen Kaur[2], Regina Ching Hua Lee[2], Chee Keng Mok[3], Patchara Phuektes[4], Li Sze Yeo[2], Vincent T.K. Chow[2], Yong Wah Tan[5], Justin Jang Hann Chu[2,3,5]
[1]Lee Kong Chian School of Medicine, Nanyang Technological University; [2]Department of Microbiology and Immunology, Yong Loo Lin School of Medicine, NUS; [3]NUS Medicine Biosafety Level 3 Core Facility, NUS; [4]Khonkaen University, Thailand; [5]Institute of Molecular and Cell Biology (IMCB), A*STAR, Singapore

Introduction: Positive-sense RNA viruses

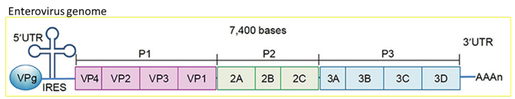
Single-stranded RNA genome (+ssRNA) acts as messenger RNA in host cells for transcription and translation during replication. All +ssRNA viruses have similar life cycles involving ER and Golgi apparatus for protein post-translation modification and/or for the formation of viral replication factories [1].

Below are examples of +ssRNA genome;

Dengue virus genome



Enterovirus genome



Human pathogens that positive-sense RNA viruses:
SARS-CoV; Dengue virus; Zika virus; Chikungunya virus; Enterovirus

These viruses are medically important as they cause hundreds of millions of life-threatening diseases yearly, including **dengue haemorrhagic fever**, **Hand, Foot, and Mouth Disease**, and **COVID19**.

Introduction: Peruvoside and its origin

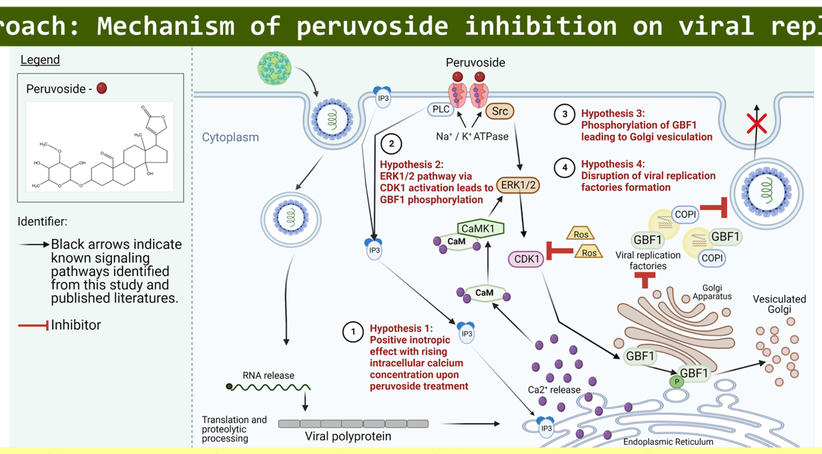
Peruvoside is a **plant-derived saponin glycoside** undergone clinical trials for the treatment of cardiac failure and arrhythmia. Similar compounds eg. digitoxigenin and ouabain are currently FDA-approved for the treatment of cardiac conditions.

These compounds function by exerting a **positive inotropic effect** on cardiac muscle cells, raising intracellular calcium concentration. They are specific inhibitors of the **sodium potassium pump (Na+/K+ ATPase)** [2] located at the plasma membrane. In the classical Src 'ionic' pathway, glycoside binds at the extracellular of the Na+/K+ ATPase α-subunit and results in a complete loss of pump activity and an eventual increase in cytosolic sodium.

Digitoxin exhibits antiviral activities against RNA virus (influenza virus) and DNA viruses such as herpes simplex virus (HSV1) and human cytomegalovirus (CMV). However, the **mechanism of action of peruvoside** as an antiviral remains largely unknown and should be deliberated.

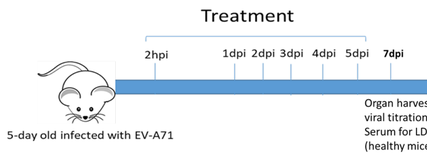
Cascabela Thevetia. The plant that peruvoside was obtained from. Adapted from www.projectnoah.org

Approach: Mechanism of peruvoside inhibition on viral replication



Approach: A schematic presentation of hypotheses that peruvoside inhibits viral replication via Src/ERK kinase cascade activation of CDK1 and GBF1 phosphorylation and phosphorylated GFB1 successively causes direct / indirect Golgi vesiculation which curtails viral replication factories formation.

Approach: In vivo model



Approach: Enterovirus A71 (EV-A71) was used as a model virus in the treatment modality. Mice were treated for 5 days post-infection and survival and clinical scoring were noted up to 20 days post-infection. Organs were harvested for viral titration on 7-days post-infection. Blood serum was harvested for LDH assay from healthy mice treated with peruvoside for 5 days.

1. Peruvoside shows broad-spectrum inhibition against RNA viruses.

Table 1: IC_{50}, EC_{50} and SI for all tested virus upon Peruvoside treatment.

Virus	Family	Species	IC_{50}	EC_{50}	SI
EV-A71 (HFM41)	*Picornaviridae*	Enterovirus A	18.63	3118	167.36
CV-A16	*Picornaviridae*	Enterovirus A	24.52	3118	127.16
CV-A6	*Picornaviridae*	Enterovirus A	4.79	3118	650.94
Echovirus 7	*Picornaviridae*	Enterovirus B	1.62	3118	1924.69
CHIKV	*Togaviridae*	Chikungunya virus	10.14	5563	548.61
ZIKV	*Flaviviridae*	Zika virus	14.55	234	16.08
DENV2	*Flaviviridae*	Dengue virus	18.01	234	12.99
SARS-CoV-2	*Coronaviridae*	Severe acute respiratory syndrome–related coronavirus	14.09	1734	123.07
MHV	*Coronaviridae*	Murine Coronavirus	20.83	5222	250.69
HSV	*Herpesviridae*	Herpes simplex virus 1	19.25	3118	161.97
H1N1	*Orthomyxoviridae*	Human influenza A	51.39	367	7.14

- IC_{50}, EC_{50} and SI for all tested viruses were determined by cell viability assay using alamarBlue® and viral titration via plaque assay. All virus infections were performed at 6 different peruvoside concentration (ranging from 10nM to 100nM).

- Peruvoside showed **broad-spectrum antiviral activity** across four families of positive-sense RNA viruses, ***Picornaviridae***, ***Coronaviridae***, ***Togaviridae*** and ***Flaviviridae***, one family of DNA virus, ***Herpesviridae***, one family of negative–sense single-stranded RNA virus, ***Orthomyxoviridae***.

2. Positive ionotropic effect with rising intracellular calcium concentration upon peruvoside treatment

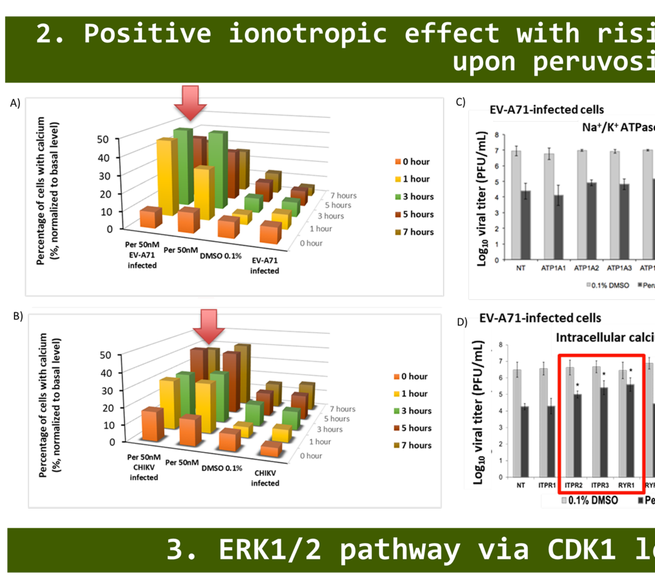


Figure 1:
(A) Intracellular Ca_{2+} reserve level upon treatment with peruvoside or 0.1% DMSO, with or without EV-A71 infection, and **(B)** with or without CHIKV infection.
(C) siRNA knockdown of selected components of the Na+/K+ ATPase signalosome and **(D)** of selected intracellular calcium channel proteins on RD cells then infected with EV-A71 (H) and treated with 50 nM peruvoside or 0.1% DMSO.

- **Intracellular calcium flux is an essential mechanism of peruvoside's antiviral activity and the calcium flux is mediated via both the Src 'ionic' pathway of extracellular calcium influx and the 'alternative' pathway of PLC/IP_3 signaling.**

3. ERK1/2 pathway via CDK1 leads to GBF1 phosphorylation

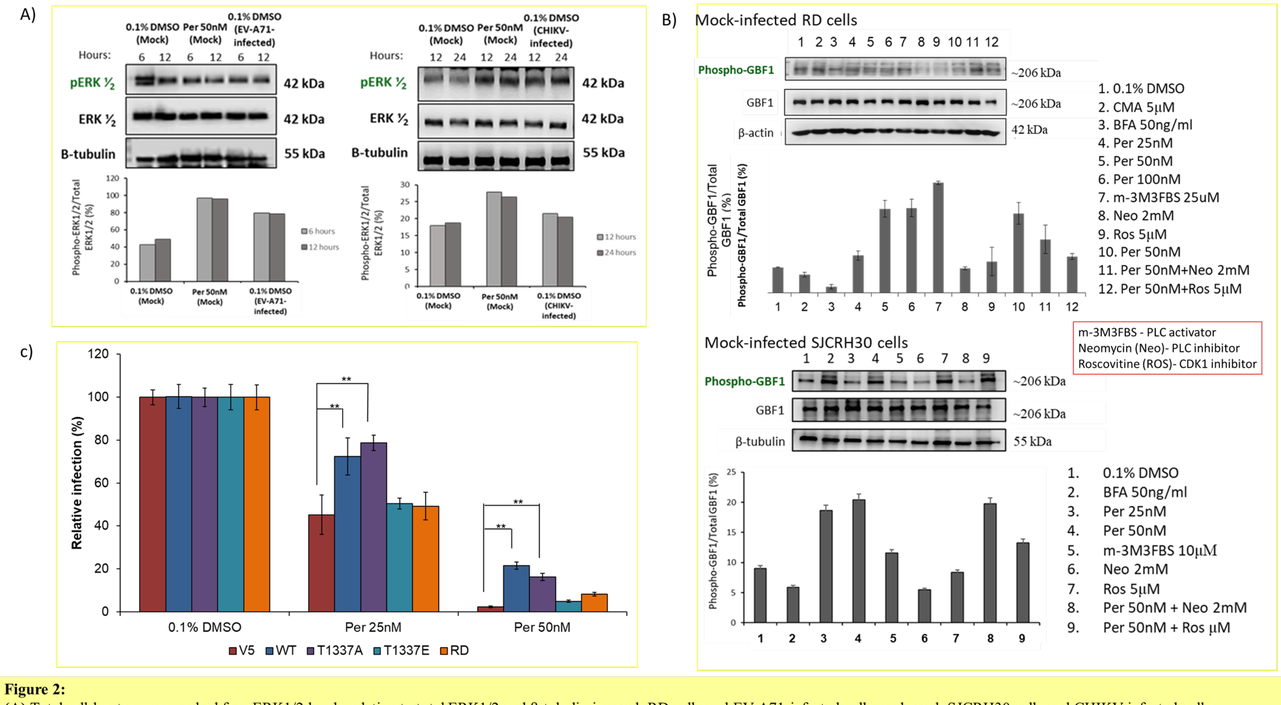


Figure 2:
(A) Total cell lysates were probed for pERK1/2 levels relative to total ERK1/2 and β-tubulin in mock RD cells and EV-A71-infected cells, and mock SJCRH30 cells and CHIKV-infected cells.
(B) Cells were treated with 0.1% DMSO (negative control), chlormadinone acetate (CMA), brefeldin A (BFA), peruvoside (Per) at different concentrations, m-3M3FBS, neomycin (Neo) and roscovitine (Ros) or in combinations and observed for GBF1 phosphorylation.
(C) Immunofluorescence was performed to determine the extent of inhibition of EV-A71 (H) infection by peruvoside in RD cells stably expressing GBF1 (wildtype, WT), non-phosphorylatable mutant (T1337A) or phosphomimetic mutant (T1337E).

- **These results revealed ERK1/2 pathway via CDK1 activation leads to GBF1 phosphorylation at Threonine 1337 (T1337A).**

3. ERK1/2 pathway via CDK1 leads to GBF1 phosphorylation

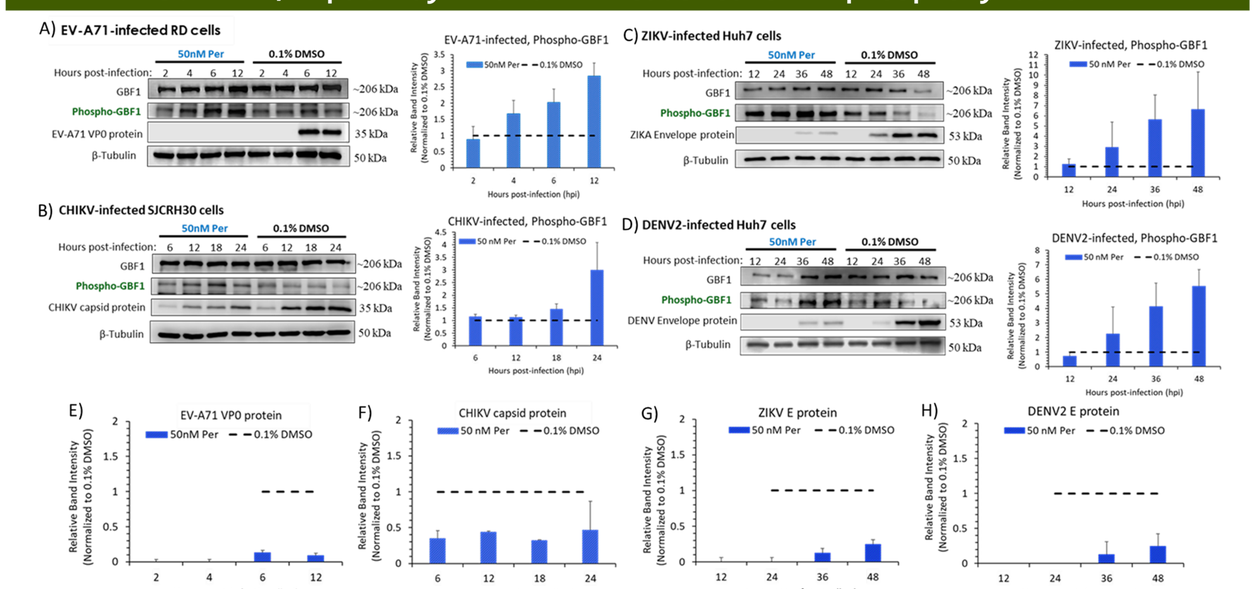


Figure 3:
Western blotting on total cell lysates of **(A)** EV-A71-infected RD cells, **(B)** CHIKV-infected SJCRH30 cells, **(C)** ZIKV-infected and **(D)** DENV2-infected Huh7 cells treated with peruvoside (Per) and 0.1% DMSO. All bar graph shows phospho-GBF1 expression normalized to 0.1% DMSO treated cells.
Quantification of band intensities was performed with Image Studio (LI-COR) for **(E)** EV-A71 VP0, **(F)** CHIKV capsid, **(G)** ZIKV envelope (E) protein, and **(H)** DENV2 E protein.

- **These results confirmed Golgi vesiculation via GBF1 phosphorylation as peruvoside's mechanism of viral RNA replication inhibition for the tested viruses.**

4. Phosphorylation of GBF1 leading to Golgi vesiculation

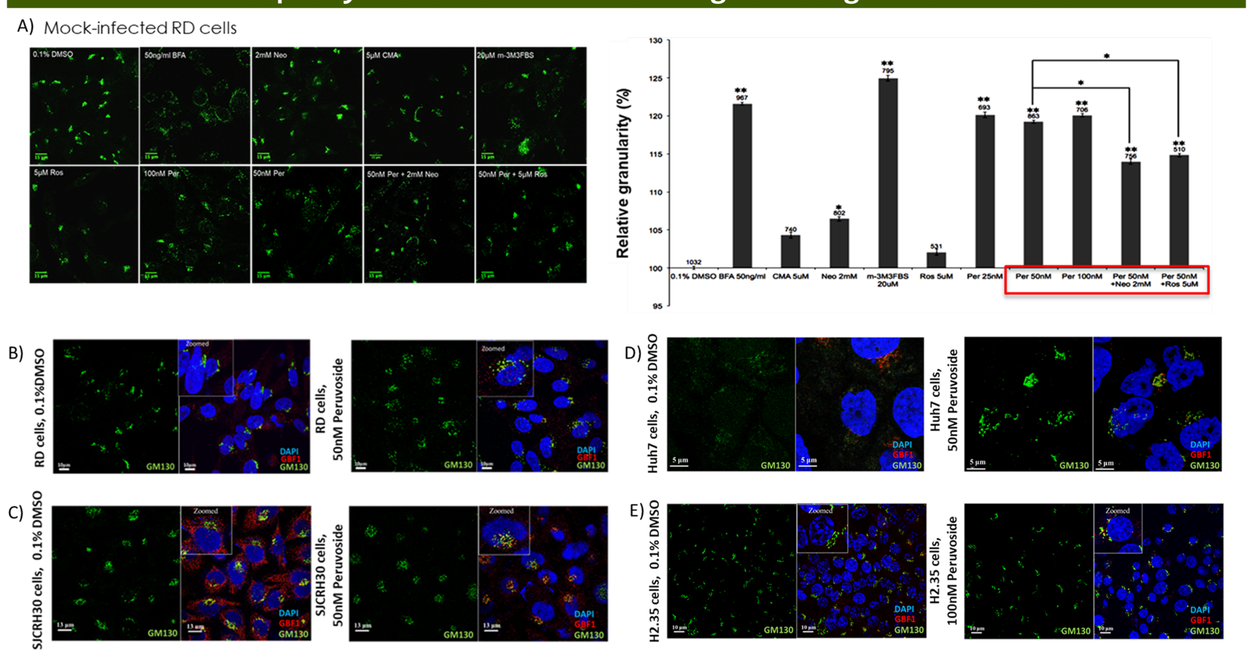


Figure 4:
(A) Immunofluorescence staining of Golgi marker (GM130) and granularity of Golgi revealed Golgi vesiculation and significantly enhanced granularity following treatment with peruvoside, BFA, and m-3M3FBS (PLC activator), while the rescue of granularity upon Ros treatment (CDK1 inhibitor) and Neo treatment (PLC kinase inhibitor) was noted. BFA was included as a positive control as it is known to cause Golgi dispersal. *p<0.05, **p<0.01.
(B) RD cells, **(C)** SJCRH30 cells, **(D)** Huh7 cells, and **(E)** H2.35 cells showed extensive colocalization of GBF1 and GM130 (Golgi marker) in the perinuclear region upon peruvoside treatment.

- **Taken together, these results portray GBF1 phosphorylation leads to Golgi vesiculation in peruvoside-treated cells.**

5. Disruption of viral replication factories formation

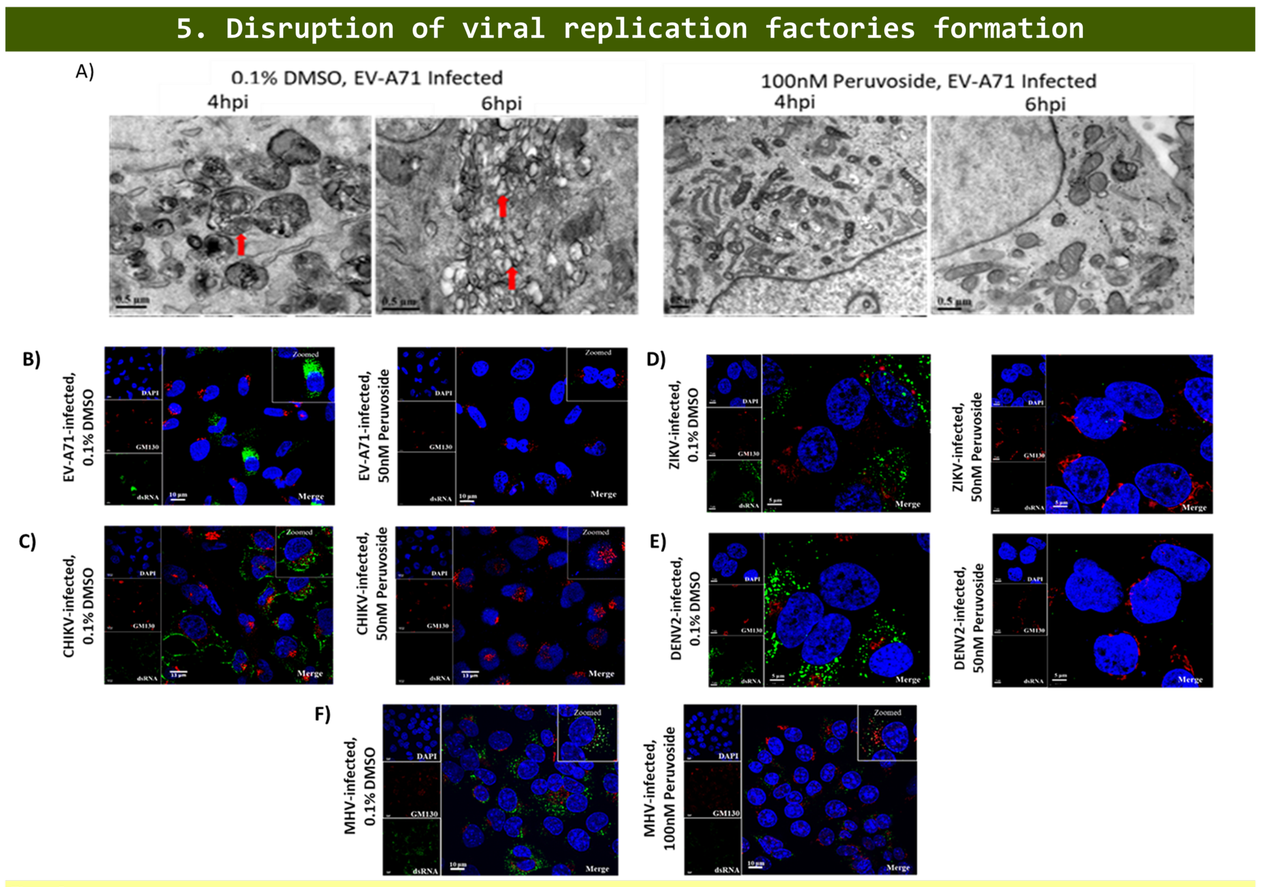


Figure 5:
(A) Electron microscopy of EV-A71 infected RD cells showed the generation of viral replication factories (arrow) that accumulated as infection progressed. EV-A71-infected and peruvoside-treated cells abolished the formation of viral replication factories.
(B) EV-A71, **(C)** CHIKV, **(D)** ZIKV, **(E)** DENV2, and **(F)** MHV infection showed Golgi stacks vesiculation within a perinuclear distribution pattern upon treatment with peruvoside. Golgi vesiculation was associated with the lower detection of viral dsRNA in cells infected with EV-A71, CHIKV, ZIKA, DENV2 and MHV when compared to 0.1% DMSO-treated cells. Viral dsRNA is produced as a replicative intermediate during RNA virus replication.

- **Inhibition of viral replication factories formation with the ramification of GBF1 and Golgi vesiculation upon peruvoside treatment was clearly observed.**

6. EV-A71 used as model virus in treatment modality

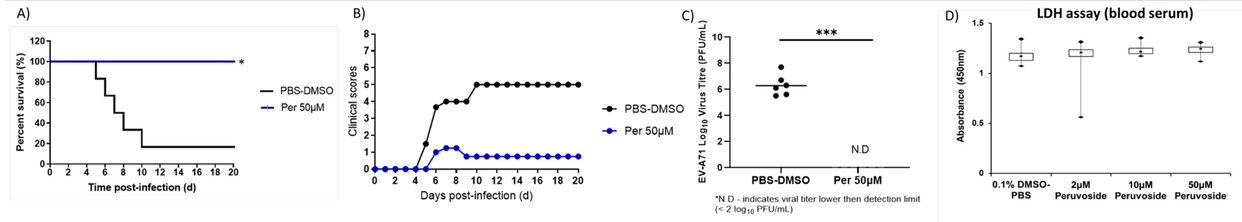


Figure 6:
(A) Survival curve of intraperitoneally injected BALB/c mice with $1x10^5$ PFU EV-A71 (HFM41) and 50μM peruvoside-treated mice monitored up to 20 days post-infection displayed a significant correlation (*p=0.0043) by log-rank (Mantel-Cox) test.
(B) Clinical scoring evaluated by symptoms of EV-A71 infected mice which includes limb paralysis, ruffled fur, inactivity, rapid breathing and weight loss.
(C) Hind-limb viral titration on 7dpi with EV-A71 and peruvoside-treated EV-A71-infected mice displayed a significant reduction of about 6 logs unit (p=0.0001).
(D) LDH assay was performed on serum collected from healthy BALB/c mice administered with 50μl of peruvoside showed no measurable difference upon peruvoside treatment.

- **Taken together, peruvoside demonstrated high potency of EV-A71 inhibition in in vivo model with no observable adverse effects.**

Conclusion

This study highlights the importance of GBF1 in viral replication. GBF1 plays a role for many viruses from remodeling the intracellular membranes to forming viral replication factories, host environment modification [3] and viral assembly/maturation processes [4]. We depictured **GBF1 phosphorylated** via **ERK1/2 activation** of the **CDK1** pathway upon peruvoside treatment.

We showed GBF1 functions could be made inaccessible to viral factors by activation of its phosphorylation triggered by peruvoside and the after effect of vesiculation of Golgi may **impair viral replication or maturation of viral protein**. By targeting host factors/processes shared by many viruses, peruvoside exhibited **inhibitory activity on a broad array of RNA viruses**. Peruvoside also showed **potent inhibition in in vivo mouse model**. These pave the way for further consideration of peruvoside as **host-directed antivirals**.

Acknowledgements

This study was supported by the following grants:
Ministry of Education Tier 2 grant (MOE2017-T2-1-078 and MOE-2017-T2-2-014), and National Research Foundation Competitive Research Programme (NRF-CRP21-2018-0004).

References:
1. Wolff G, Melia CE, Snijder EJ, Bárcena M. Double-Membrane Vesicles as Platforms for Viral Replication. Trends in Microbiology 2020. 2. Sandtner W, Egwolf B, Khalili-Araghi F, Sanchez-Rodriguez JE, Roux B, Bezanilla F, et al. Ouabain Binding Site in a Functioning Na+/K+ ATPase. Journal of Biological Chemistry 2011;286:38177-83. 3. Zhang L, Hong Z, Lin W, Shao RX, Goto K, Hsu VW, et al. ARF1 and GBF1 generate a PI4P-enriched environment supportive of hepatitis C virus replication. PLoS One 2012;7:e32135. 4. Solignat M, Gay B, Higgs S, Briant L, Devaux C. Replication cycle of chikungunya: a re-emerging arbovirus. Virology 2009;393:183-97.