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Author(s)	PAULINA DUHITA ANINDITA
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Development and Implementation of a Compound Screening Assay to Identify Antivirals against Rabies Virus

(狂犬病ウイルスに対する抑制作用を有する化合物のスクリーニングアッセイ法の開発と化合物の探索**)**

Paulina Duhita ANINDITA

CONTENTS

List of Abbreviations 1

General Introduction 4

Chapter I

Generation of Recombinant Rabies Viruses encoding NanoLuc Luciferase

Introduction 7

Materials and Methods 9

Cells 9

Plasmids 9

Propagation and recovery of rRABVs 10

Virus titration by focus forming assay 10

Luciferase assay 11

Infection experiments with rRABVs 11

Immunoblotting 12

Results 13

Construction of rRABVs encoding NanoLuc 13

Growth kinetics and stability of NanoLuc expression from rRABVs 17

Measurement of antiviral activities using rRABVs 20

Discussion 24

Summary 28

Chapter II

Investigation of Anti-Rabies Virus Activity of Ribavirin-Related Compounds

Introduction 29

Materials and Methods 32

Cells 32

Viruses 32

Compound examination 32

Luciferase assay 33

Focus forming assay 33

Cell viability assay 33

Plasmids 34

RABV minigenome assay 35

Immunofluorescence assay 35

Time-of-addition assays 35

Quantitative real-time reverse transcription PCR (qRT-PCR) assay 36

Results 38

Antiviral activity and cytotoxicity of EICAR, EICNR, BICAR, mizoribine and ribavirin 37

Confirmation of antiviral activity of EICAR and EICNR 43

EICAR and EICNR inhibit the viral post-entry stage 45

EICAR and EICNR inhibit viral transcription and genome replication 49

Discussion 52

Summary 55

Conclusions 57

Acknowledgements 59

References 61

List of Abbreviations

BICAR	5-buthynyl-1-β-D-ribofuranosylimidazole-4-carboxamide
CC_{50}	concentration required to reduce cell viability of a given cell line by 50%
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CPE	cytopathic effect
CVS	challenge virus standard
DMEM	Dulbecco's modified MEM
DMSO	dimethyl sulfoxide
EC_{50}	concentration required to reduce NanoLuc activity of a given rRABV-infected-cell line by 50%
EICAR	5-ethynyl-1-β-D-ribofuranosylimidazole-4-carboxamide
EICNR	5-ethynyl-1-β-D-ribofuranosylimidazole-4-carbonitrile
EMEM	Eagle's minimum essential medium
FAM	carboxyfluorescein
FBS	fetal bovine serum
FFU/ml	focus forming unit per millilitre
FITC	fluorescein isothiocyanate
G	glycoprotein
h	hour
HA	hemagglutinin
HDV	hepatitis delta virus
hpi	hour post-infection

HRP	horseradish peroxidase
IGR	intergenic region
IMP	inosine-5’-monophosphate
kb	kilobase
kDa	kilodalton
L	RNA-dependent RNA polymerase
M	matrix protein
MEM	minimum essential medium
MGB	minor groove binding
mins	minutes
MOI	multiplicity of infection
mRNA	messenger Ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	nucleoprotein
NA	mouse neuroblastoma
NanoLuc	NanoLuc luciferase
Nluc	non-secreted NanoLuc
NP-40	nonidet P-40
nts	nucleotides
ORF	open reading frame
P	phosphoprotein
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PEP	post-exposure prophylaxis
qRT-PCR	quantitative real-time reverse transcription PCR

RABV	rabies virus
RLU	relative light unit
rRABV	recombinant RABV
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
secNluc	secreted NanoLuc
SK-N-SH	human neuroblastoma
WHO	World Health Organization
WT	wild-type

General Introduction

Rabies is an acute and invariably fatal encephalomyelitis, resulting from infection by any of the viruses in the genus *Lyssavirus*, family *Rhabdoviridae* and the principal lyssavirus that causes this devastating neurological disease is rabies virus (RABV) [1]. Rabies is mainly transmitted through the bite of rabid animals even though evidence for airborne routes [2, 3] and organ transplantation [4, 5] has been reported for rabies transmission in a limited number of cases. Rabies can infect a wide range of susceptible mammalian hosts; however, rabies cases in humans are predominantly related to transmission from rabid dogs and the number of stray dogs present in the endemic areas since most of the cases arise from dog bites [6]. Rural areas in Asian and African countries, with a large number of free-roaming dogs, suffer the highest disease burden from rabies [7]. World Health Organization (WHO) estimates that 60,000 people die from rabies with more than 15 million people receiving post-exposure prophylaxis (PEP) worldwide annually (WHO Rabies Fact Sheet 2016, accessed June 10, 2017, from http://www.who.int/mediacentre/factsheets/fs099/en/).

RABV is an enveloped virus containing a single-stranded, nonsegmented, negative sense RNA genome of ~12 kb composed of five open reading frames (ORFs) encoding: nucleoprotein, N; phosphoprotein, P; matrix protein, M; glycoprotein, G and the RNA-dependent RNA polymerase, L. Each RABV gene is flanked by short regulatory sequences, referred to as start and stop signals, that regulate transcription of the contiguous open ORF [8, 9]. Between each RABV gene, there is an intergenic region (IGR) with differing nucleotide (nt) lengths: 2 nts between N and P (N/P) IGR, 5 nts between P and M (P/M) IGR, 5 nts between M and G (M/G) IGR and 24-29 nts between G and L (G/L) IGR. These nucleotide sequences are neither transcribed into mRNA nor translated into protein. The length of the nucleotide sequence in the RABV IGR has been reported to contribute to transcriptional

attenuation of the downstream viral gene and to be involved in regulating viral protein expression [10].

Once RABV is transmitted to a susceptible individual, about 80% will develop an encephalitic rabies, a classical form of rabies which is characterized by the occurrence of general arousal and hyperexcitability that can be accompanied with hydrophobia, aerophobia and sensitivity towards bright lights or loud sounds; whereas the remainder of individuals (~20%) will develop a paralytic rabies which is characterized by general muscle weakness [1, 11]. Both forms of rabies have similar clinical stages that include: incubation period which length depends on the bite site, prodromal period, acute neurological signs, coma and, finally, death [12].

Rabies vaccine is available both as a preventive measure and included in a PEP regimen given immediately after a person is bitten by a rabid animal, together with wound cleaning and administration of rabies immunoglobulin [13]. A number of drugs, such as, ribavirin, amantadine, interferon alpha and ketamine have been employed to treat symptomatic rabies patients; however, clinical efficacy has not been demonstrated, with few survival cases recorded [14-16]. Thus, novel antiviral drugs to treat symptomatic rabies patients are urgently needed to mitigate the devastating neurological sequelae associated with this high-consequence pathogen.

A limited number of studies involving screening of small chemical compounds have been performed on RABV to find novel antivirals [13, 17-19]. The assays employed in the screening depended on the target of the small compounds in the RABV life cycle. A cell-free protein synthesis system has been developed to identify small compounds that inhibit the assembly of the RABV ribonucleoprotein capsid [19]. Cell-based screening assays have also been successfully utilized to identify compounds which target viral replication and protein synthesis steps of the viral life cycle [20-22].

In chapter I of this thesis, recombinant RABVs (rRABVs) encoding NanoLuc luciferase (NanoLuc) were generated to enable a screening strategy to identify compounds with antiviral activity against RABV. The effectiveness of rRABVs to represent the replication of wild-type (WT) RABV was experimentally validated and this allowed the rapid quantification of virus replication compared to more laborious and time-consuming conventional assays. Moreover, a direct comparison between rRABVs harbouring 2-29 nt IGR lengths revealed that shorter IGR sequences upstream of reporter genes were associated with higher expression levels and that the relative distance of the reporter gene to the promoter site also played a significant role in robust reporter gene expression. NanoLuc was also found to be stably expressed over the course of ten serial passages and the rRABVs were sensitive to ribavirin, a nucleoside analog antiviral compound known to inhibit RABV replication *in vitro*, demonstrating proof-of-concept of this methodological approach [23, 24] .

In chapter II, anti-RABV activity of ribavirin-related compounds were investigated employing rRABV generated in Chapter I. It was determined that several ribavirin analogs possessed greater antiviral activity when compared to ribavirin to inhibit RABV replication *in vitro*. Further characterization of the mechanism of action of these compounds suggested that inhibition of viral protein expression and genome replication mediated the antiviral effects elicited by these drugs.

Chapter 1

Generation of Recombinant Rabies Viruses encoding NanoLuc Luciferase

Introduction

Rabies is an invariably fatal viral infection of the central nervous system (CNS) which occurs worldwide, and human deaths have been frequently reported, particularly in rural areas of Asian and African countries [6, 7, 25]. While vaccination is protective against RABV infection, an effective and safe PEP has also been established to treat patients bitten by rabid animals and it must be administered before the onset of symptoms [11, 26]. Once symptoms have developed, there are no effective therapeutic measures that can inhibit disease progression leading to a case fatality rate approaching 100% [11]. Thus, novel antivirals are urgently required to treat symptomatic RABV-infected patients and can potentially be discovered by screening of compound libraries.

Classical methods, such as plaque assays, have been used in compound screening to quantify viral titers of compound-treated permissive cells; however, this type of assay approach is laborious and time-consuming. RABV infection does not usually produce cytopathic effects (CPEs) in some cell lines [27-29]. Therefore, RABV infection cannot be readily detected using cell viability assays that are commonly employed in compound screening. In contrast, viruses encoding reporter genes have been generated for screening of small compounds, since antiviral efficacy and viral replication can be reflected by the expression of the reporters [30-32]. Reporters that have luminescence properties possess

greater advantages in the screening of small compounds because these reporters have lower background signals and higher sensitivity compared to fluorescence reporters [33]. Several studies have also described rRABVs encoding fluorescent or luminescent proteins for various applications [34-39].

To address the technical limitations of screening of small compounds for RABV, rRABVs encoding NanoLuc, a small (19 kDa) luminescent reporter protein which produces brighter luminescence compared to *Renilla* and firefly luciferase [40], were generated. Luminescent properties of NanoLuc provide more sensitivity, higher accuracy and lower background levels compared to fluorescent protein, such as green fluorescent protein [41]. Previous studies have also suggested that NanoLuc reporter viruses are highly sensitive in measuring viral replication *in vitro* and *in vivo* and can be used in small compound screening assays [42, 43]. In the present study, the rRABV luciferase activity was detectable in cell lysates and culture supernatants from RABV-infected cells dependent on the nature of the NanoLuc (non-secreted or secreted NanoLuc). Characterization of the rRABVs showed that the luciferase activity could be stably detected in RABV-infected cells and positively correlated with rRABV inoculum. Finally, the rRABVs was employed to validate the antiviral activity of ribavirin, demonstrating their utility for compound screening assays.

Materials and Methods

Cells

Human neuroblastoma (SK-N-SH) cells obtained from the Riken BRC Cell Bank (Tsukuba, Japan) were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). Mouse neuroblastoma (NA) cells were also maintained under the same conditions as that of SK-N-SH cells. Baby hamster kidney cells stably expressing T7 RNA polymerase (BHK/T7-9) [44] were maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% tryptose phosphate buffer and 10% FBS. All cells were grown in an incubator at 37^{o}C with 5% CO_2 atmosphere.

Plasmids

A plasmid vector containing the full-length cDNA of challenge virus standard (CVS) RABV strain, pCVS, was generated as a backbone (Fig. 1A). Briefly, pCVS was linearized by restriction enzyme digestion with BlnI and SpeI for insertion of the reporter gene in N/P IGR or SalI for insertion in G/L IGR. The DNA fragments encoding non-secreted NanoLuc (Nluc) from pNL2.1 [Nluc/Hygro] (Promega, Madison, WI) or secreted NanoLuc (secNluc) from pNL2.3 [secNluc/Hygro] (Promega) were then amplified by polymerase chain reaction (PCR) employing oligonucleotide primers incorporating transcription start and stop signal sequences from the RABV N and P genes for N/P IGR insertion or M and G genes for G/L IGR insertion at both 5' and 3' ends of the *NanoLuc* gene fragments to that is essential for the transcription of the genes. Partial RABV gene fragments were also generated by PCR amplification. Plasmids encoding NanoLuc were then generated by ligating linearized pCVS and inserts (partial RABV genes and *NanoLuc* genes) using the In-Fusion HD Cloning Kit (Takara Bio, Kusatsu, Japan). The resulting plasmids containing each type of NanoLuc, either

in the N/P IGR (Fig. 1B) or G/L IGR (Fig. 1C), were transformed into *Escherichia coli* (strain HST08) (Takara Bio). Constructs were verified by DNA sequencing and the rRABV fusions were designated as pCVS-Nluc (N-P), pCVS-secNluc (N-P), pCVS-Nluc (G-L) and pCVS-secNluc (G-L).

Propagation and recovery of rRABVs

BHK/T7-9 cells monolayers grown in 12-well plates were individually transfected with 1.2 μg of either pCVS-Nluc (N-P), pCVS-secNluc (N-P), pCVS-Nluc (G-L) or pCVS-secNluc (G-L), together with helper plasmids: pT7IRES-RN (0.2 μg), -RP (0.05 μg) and -RL (0.1 μg). The transfected cells were incubated for 4 days at 37^{o}C and thereafter, rRABVs were harvested from culture supernatants of the transfected BHK/T7-9 cells. Recovered rRABVs were then inoculated to fresh murine NA cells and incubated for another 5 days. One day after passage, the infectious rRABVs were detected by immunostaining using anti-RABV N protein antibody conjugated with fluorescein isothiocyanate (FITC) (Fujirebio Diagnostics, Inc., Malvern, PA), as previously described [45]. Following the incubation, rRABVs were harvested from culture supernatants and kept at -80^{o}C as rRABV stock. Stock titers of rRABVs were determined by focus forming assays.

Virus titration by focus forming assay

Focus forming assay was performed by inoculation of rRABVs into NA cells in 24-well plates with a 10-fold dilution series of each rRABV in MEM. Following incubation for 1 hour (h) at 37^{o}C on a plate shaker, an overlay medium containing MEM (supplemented with 5% FBS; penicillin, 100 U/ml; streptomycin, 100 mg/ml and 0.5% methylcellulose) was added to cells. Foci of rRABVs were detected using anti-RABV N protein antibody

(Fujirebio Diagnostics, Inc.) after 3 days of incubation. The number of foci was counted and the virus titer in focus forming unit per millilitre (FFU/ml) was determined.

Luciferase assay

The NanoLuc activity was measured using the Nano-Glo luciferase assay kit (Promega) and GloMax 96 microplate luminometer (Promega). Whole cell lysates were used to measure NanoLuc activity from cells infected with rRABVs encoding Nluc, whereas supernatants were employed to measure NanoLuc activity from rRABVs encoding secNluc, following the protocol recommended by the manufacturer. The measured NanoLuc activity was expressed in relative light units per second (RLU/sec).

Infection experiments with rRABVs

All infection experiments were performed in the Biosafety Level-3 facility, Research Center for Zoonosis Control, Hokkaido University. For measurement of virus growth kinetics, NA cells were infected with rRABVs at a multiplicity of infection (MOI) of 1 where the MOI was calculated as FFU/cell. The supernatants were harvested at 1 h post-infection (hpi) and subsequent times followed by focus formation assays to determine the virus titer.

For measurement of the kinetics of NanoLuc activities, SK-N-SH cells seeded in 96-well black clear-bottom plates (Thermo Fisher Scientific, Lafayette, CO) were infected with rRABVs at an MOI of 1. After 1 hpi, the cells were washed with phosphate buffer saline (PBS) and fresh medium was added to the cells prior to incubation at 37^{o}C with 5% CO_2. The cells or supernatants were harvested at 0, 8, 24, 48, 72, and 96 hpi.

For analysis of antiviral activity of ribavirin (Wako, Osaka, Japan) against RABV, SK-N-SH cells were seeded in 96-well black clear-bottom plates 1 day prior and were infected with either CVS-Nluc (N-P) or CVS-secNluc (N-P) at an MOI of 0.1 for 1 h at 37^{o}C. After

washing the cells with PBS, fresh medium containing serial dilutions of ribavirin were added. Serially diluted ribavirin was prepared by diluting the stock of ribavirin in dimethyl sulfoxide (DMSO) in cell culture medium at final concentrations of 200, 100, 50, 25, 12.5 and 6.25 μM. The NanoLuc activity in either cell lysates or supernatants of infected cells were measured at 24 hpi.

Immunoblotting

Immunoblotting was performed as previously described with modifications [46]. SK-N-SH cells seeded in 12-well plates were inoculated with each rRABV and harvested at 24 hpi. Cells were suspended in Nonidet P-40 (NP-40) lysis buffer [1% NP-40 (Sigma, St. Louis, MO), 20 mM Tris-HCl (pH 7.5), 150 mM NaCl] supplemented with complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Cell lysates were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were then transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA). The membrane was incubated with rabbit anti-Nluc (provided by Promega), mouse monoclonal anti-RABV N (Hytest Ltd., Turku, Finland) or mouse anti-actin (Chemicon, Millipore, Temecula, CA). Immune complexes were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (Biosource International, Camarillo, CA), Immunostar LD Chemiluminescence Reagent (Wako), and a VersaDoc 5000MP (Bio-Rad, Hercules, CA).

Results

Construction of rRABVs encoding NanoLuc

Transcription of viral genes in nonsegmented, negative-stranded RNA viruses has been previously shown to be affected by both the gene order and the dissociation of the viral polymerase at each gene border resulting in a gradient of gene transcripts [47, 48]. More studies inserting reporter genes into the RABV G/L IGR exist [34-37, 49] than studies investigating the effects of the insertion of reporters into the N/P IGR [38, 39]. The rRABVs encoding NanoLuc inserted in either N/P IGR or G/L IGR were generated to compare the expression of reporter genes between those two sites.

It has also been reported that the longer G/L IGR (24-29 nts) contributed to severe downregulation of the RABV L gene, resulting in lower levels of expression of the L protein; however, when the G/L IGR was replaced with a shorter N/P IGR (2 nts), the RABV L gene was upregulated with higher expression of the L protein [10]. Thus, to drive optimal expression of NanoLuc, addition of short sequence derived from the N/P IGR upstream of the *NanoLuc* gene was investigated to achieve equivalent NanoLuc expression levels between different insertion sites. Different types of NanoLuc (secreted or non-secreted) were also employed to obtain an optimal expression between types of NanoLuc that differ in the expression system and insertion site.

The virus constructs were generated by ligation of PCR-amplified fragments of NanoLuc and RABV genes with linearized pCVS (Fig. 1A-C). Expression of the inserted NanoLuc gene was achieved by addition of the transcription start and stop signals at the 5' and 3' ends of the NanoLuc gene. The fragments consisting of 2 nts derived from the N/P IGR were inserted upstream of the start signal to enhance expression of the NanoLuc insert. Four different rRABVs encoding Nluc or secNluc inserted into the N/P IGR or G/L IGR were

generated by using BHK/T7-9 cells transfected with either pCVS-Nluc (N-P), pCVS-secNluc (N-P), pCVS-Nluc (G-L) or pCVS-secNluc (G-L). These rRABVs were designated as: CVS-Nluc (N-P), CVS-Nluc (G-L), CVS-secNluc (N-P) and CVS-secNluc (G-L). NanoLuc activities of all four rRABVs were detected in cell lysates or supernatants following transfection (Fig. 1D), demonstrating the transcription of the *NanoLuc* gene by flanking the Nluc and secNluc sequences with transcriptional start and stop signals. Even though the NanoLuc sequence inserted in the G/L IGR was also flanked by the N/P IGR sequence instead of the original G/L IGR sequence at the 5' region to determine whether this would increase NanoLuc expression, luciferase activity derived from insertion in the G/L IGR produced a lower signal compared to those of the N/P IGR either in the cell lysates of CVS-Nluc (N-P) and CVS-Nluc (G-L) or in the supernatants of CVS-secNluc (N-P) and CVS-secNluc (G-L) ($p<0.05$) (Fig. 1D).

Expression of NanoLuc was also examined by immunoblotting with an antibody against NanoLuc using whole cell lysates of SK-N-SH cells inoculated with either wild-type CVS (CVS-WT), CVS-Nluc (N-P) or CVS-Nluc (G-L). The expression of NanoLuc was detected from cell lysates of CVS-Nluc (N-P)- and CVS-Nluc (G-L)-inoculated cells (Fig. 1E).

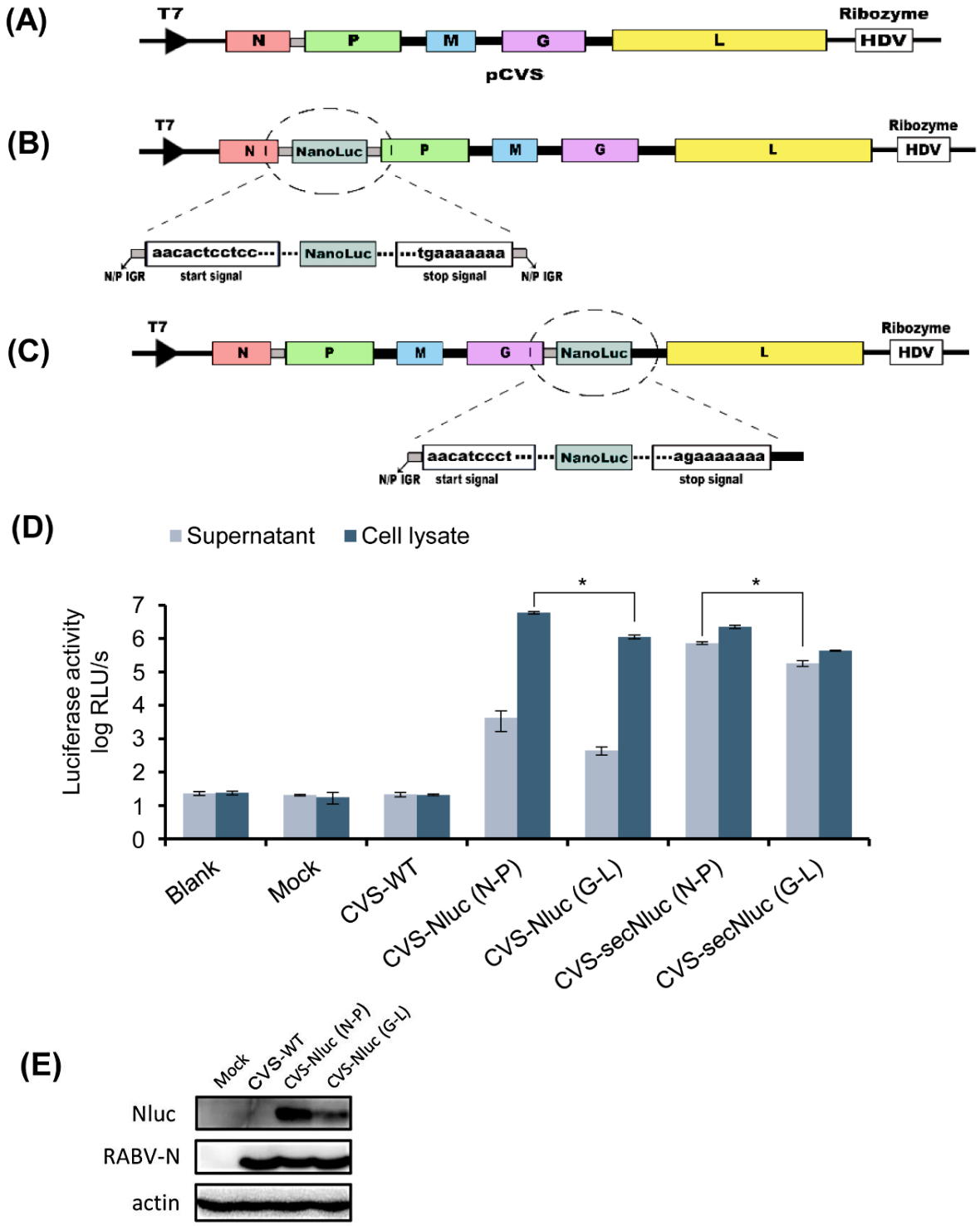

Fig. 1. Schematic representation of the construction of rRABVs. (A) Scheme of construction of plasmids encoding wild-type RABV challenge virus standard strain (pCVS). (B and C) *NanoLuc* genes were inserted into the N/P intergenic region (IGR) (B) or the G/L IGR (C) of pCVS. Transcriptional start and stop signals were inserted at the 5' and 3' ends of the NanoLuc sequence, respectively, to ensure NanoLuc expression. All IGRs are indicated by black bars except for the N/P IGR which is indicated by grey bars. (D) NanoLuc activities

were detected in the cell lysates or supernatants SK-N-SH inoculated with each rRABV at 24 hpi. Similar results were obtained from another two separate experiments performed in triplicate. Error bars indicate standard deviation. Asterisks indicate significant differences analyzed by Student's *t*-test ($p<0.05$). (E) Expression of NanoLuc protein in whole cell lysates of SK-N-SH cells inoculated with each indicated rRABVs was examined by immunoblotting with specific anti-Nluc (Nluc), anti-RABV N (RABV-N) and anti-β-actin (actin) antibodies.

Growth kinetics and stability of NanoLuc expression from rRABVs

To examine the growth kinetics of each rRABV, NA cells were infected with either rRABV or CVS-WT and virus titers in the supernatants were determined by focus forming assays at each time point. The titers of all rRABVs in the culture supernatants were within 1 $\log_{10}$ compared with those of CVS-WT until 48 hpi (Fig. 2A and B), suggesting that growth of all rRABVs was similar as that of CVS-WT. After CVS-WT reached its maximum titer at 48 hpi, a decrease of CVS-WT titer was observed at 72 and 96 hpi. Titers of all rRABVs reached their maximum levels at 72 hpi.

The NanoLuc activities from rRABVs were also examined in SK-N-SH cells inoculated with each rRABVs encoding NanoLuc. Nluc or secNluc was detected starting at 8 hpi. The rRABVs encoding NanoLuc from the N/P IGR insertion produced significantly higher NanoLuc activities than rRABVs encoding NanoLuc from the G/L IGR insertion (Fig. 2C and D) at 8 and 24 hpi ($p<0.05$).

To confirm the stability of NanoLuc expression from rRABVs, the rRABVs were serially passaged in NA cells for a total of ten passages. The NanoLuc activities could be detected in SK-N-SH cells inoculated with each rRABV stock from passages 1, 4, 7 and 10 (Fig. 2E and F), suggesting that the transfected Nluc or secNluc cassettes were maintained without apparent loss of activities. The rRABVs encoding Nluc or secNluc at N/P IGR produced significantly higher luciferase activities than rRABVs encoding Nluc or secNluc at the G/L IGR throughout ten passages ($p<0.05$; Fig. 2E and F).

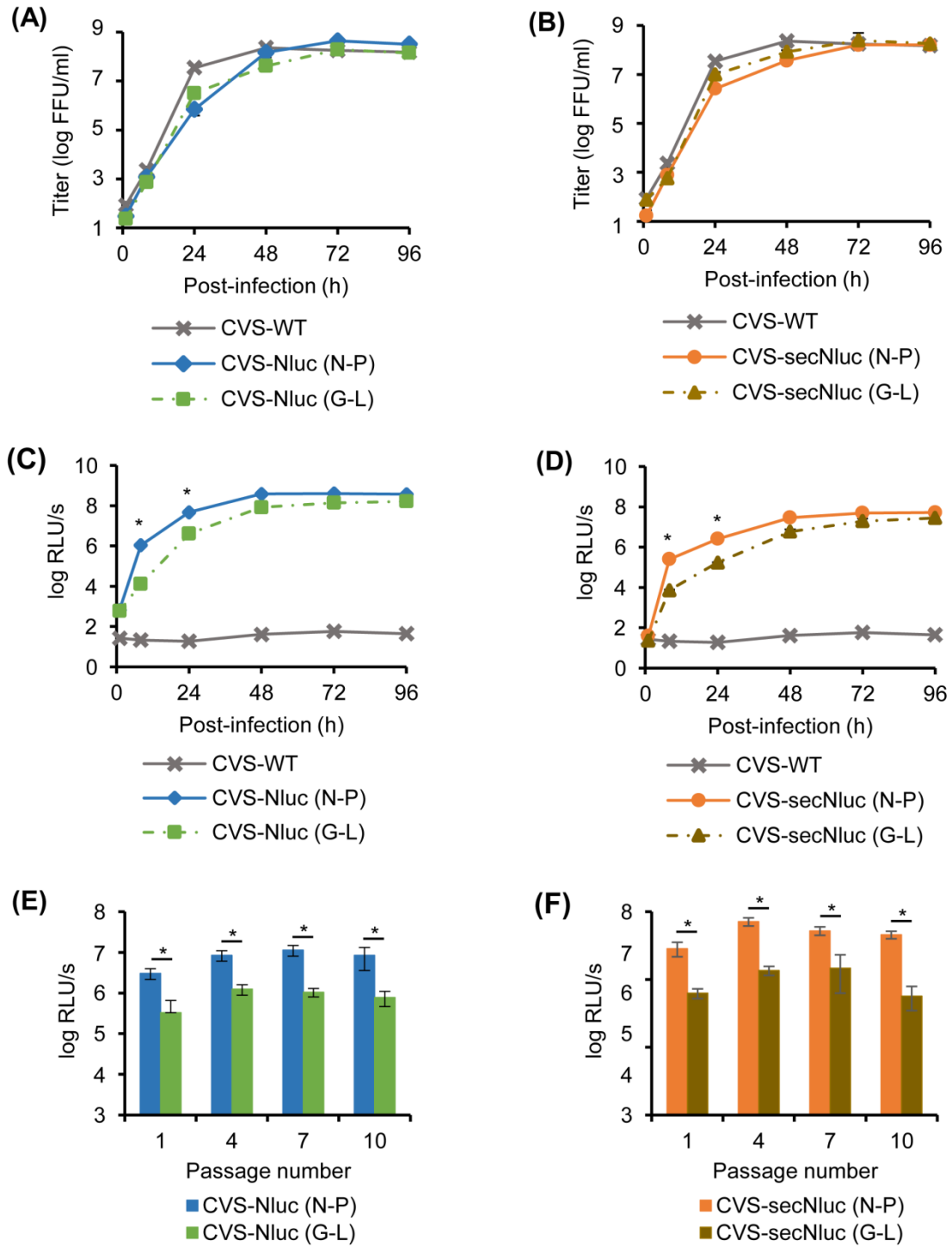

Fig. 2. Growth kinetics, NanoLuc activities and stability of NanoLuc expression from rRABVs. (A and B) Growth curves of the CVS-WT and rRABVs encoding NanoLuc. Murine NA cells were infected with each of the indicated viruses at an MOI of 1. Supernatants were then harvested at indicated time points for focus forming assays to measure the virus titer. (C and D) NanoLuc activities of rRABVs encoding NanoLuc. NanoLuc activities were measured from cell lysates for Nluc or supernatants for secNluc at

indicated time points after inoculation of each rRABV into SK-N-SH cells at MOI of 1. All data shown are representative of at least two independent experiments performed in triplicate. Mean and standard deviation are shown. Asterisks indicate significant differences between NanoLuc activities derived from rRABVs encoding NanoLuc in N/P IGR and G/L IGR at corresponding time points, analyzed by Student's *t*-test ($p<0.05$). (E and F) rRABVs encoding Nluc and secNluc were serially passaged in NA cells. Supernatants from the passages 1, 4, 7 and 10 were harvested and inoculated to SK-N-SH cells (MOI = 0.1). NanoLuc activities were then measured from cell lysates for Nluc or supernatants for secNluc at 24 hpi. The data are shown as representative of two independent experiments performed in triplicate. Mean and standard deviation are shown. Asterisks indicate significant differences between NanoLuc activities derived from rRABVs encoding NanoLuc in N/P IGR and G/L IGR from each passage, analyzed by Student's *t*-test ($p<0.05$).

Measurement of antiviral activities using rRABVs

To examine the relationship between NanoLuc expression and rRABV infectivity, the correlation between the input of rRABVs and NanoLuc activities were evaluated. Since rRABVs encoding Nluc and secNluc that were inserted in the N/P IGR showed higher luciferase activity, these recombinant viruses were the focus in further studies. SK-N-SH cells were inoculated with either CVS-Nluc (N-P) or CVS-secNluc (N-P) with differing amounts of input virus: MOI of 0.01, 0.1, 1 and 10. NanoLuc activities were measured at 16 hpi. The NanoLuc activities correlated with virus inoculum within 1 $\log_{10}$ difference that reflected the 10-fold difference in input virus for CVS-Nluc (N-P) (Fig. 3A) and CVS-secNluc (N-P) (Fig. 3B).

The antiviral activity of the nucleoside analog ribavirin against RABV was examined employing the rRABVs to further assess the usefulness of rRABV in demonstrating antiviral activity of compounds. SK-N-SH cells were infected with either CVS-Nluc (N-P) or CVS-secNluc (N-P) at an MOI of 0.1 for 1 h. Thereafter, fresh culture medium containing serially diluted ribavirin was added to the cells after washing with PBS. NanoLuc activities were measured at 24 hpi. The NanoLuc activities from cell lysates of CVS-Nluc (N-P)-infected cells were found to be gradually decreased concomitantly with increasing ribavirin concentration (Fig. 4A). A similar decrease of luciferase activity from the supernatants of CVS-secNluc (N-P)-infected cells was also observed (Fig. 4B). The EC_{50} values of ribavirin were 12 μM and 15 μM for CVS-Nluc (N-P) and CVS-secNluc (N-P), respectively and the inhibition of rRABV replication was in a dose-dependent manner.

The antiviral activity of ribavirin was also determined using the focus forming assay. The virus titer was reduced in a comparable manner with the luciferase activity of CVS-Nluc (N-P) (Fig. 4C) and CVS-secNluc (N-P) (Fig. 4D). The calculated EC_{50} values were 15 μM

for CVS-Nluc (N-P) and 12 µM for CVS-secNluc (N-P), and the EC_{50} values were in the same range (12-15 µM) of those calculated from the NanoLuc measurements.

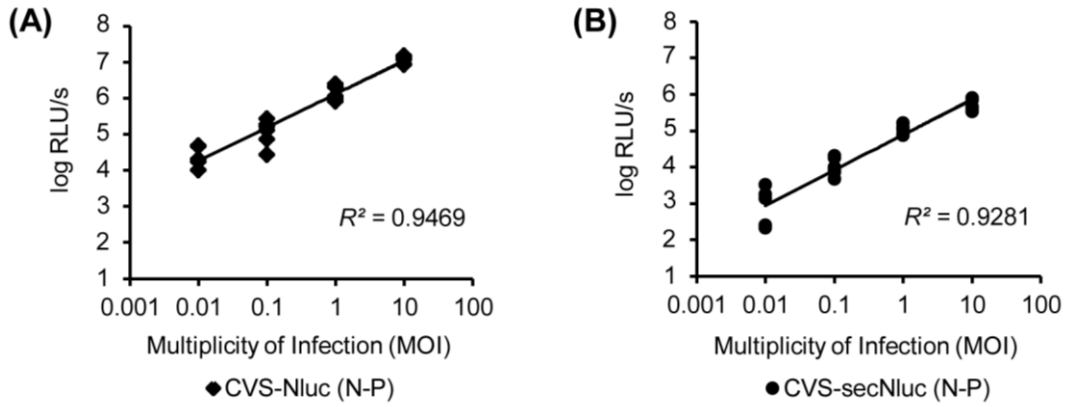

Fig. 3. Correlation between NanoLuc activities and input rRABVs. SK-N-SH cells were inoculated with CVS-Nluc (N-P) (A) or CVS-secNluc (N-P) (B) at indicated amount of input viruses. NanoLuc activities were then measured from cell lysates for CVS-Nluc (N-P) or supernatants for CVS-secNluc (N-P) at 16 hpi. The R^2 values of the linear regression analysis shown in the graph from two independent experiments performed in triplicate.

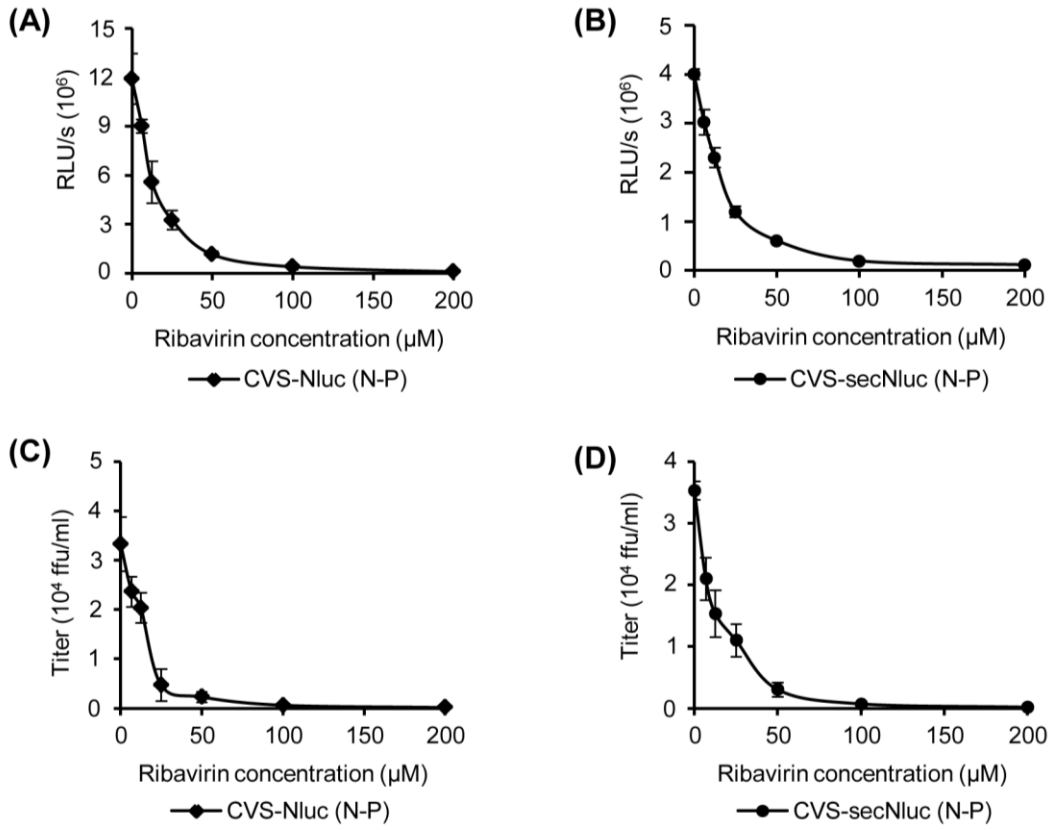

Fig. 4. Measurement of antiviral activity of ribavirin against rRABVs. (A-D) SK-N-SH cells in 96-well plates were inoculated with CVS-Nluc (N-P) (A and C) or CVS-secNluc (N-P) (B and D) at an MOI of 0.1 in the presence of the indicated concentrations of ribavirin. (A and B) NanoLuc activities were measured from cell lysates for CVS-Nluc (N-P) or supernatants for CVS-secNluc (N-P) at 24 hpi. (C and D) The effect of ribavirin against rRABV was measured by focus forming assay on NA cells using samples collected before the measurement of NanoLuc activity. All shown data are representative of three independent experiments performed in triplicate. Mean and standard deviation are shown.

Discussion

rRABVs encoding Nluc and secNluc inserted in either N/P or G/L IGRs were generated and growth rates of all rRABVs were shown to reach comparable titers to WT RABV at 72 hpi. NanoLuc activities of all rRABVs gradually increased across time points and reached plateaus at 72 hpi. All constructs of the rRABVs contained the N/P IGR sequence upstream of the *NanoLuc* gene regardless of the insertion site. The N/P IGR sequence consists of 2 nts that had previously been shown to drive optimal transcription of a reporter gene in a minigenome system when compared with other longer IGR sequences and was also associated with higher rates of RABV L mRNA synthesis from rRABVs with 2 nts N/P IGR sequences compared with rRABV carrying 24 nt G/L IGR sequences [10]. However, despite harboring identical N/P IGRs upstream of the NanoLuc sequence, higher NanoLuc activities were observed from rRABVs encoding Nluc or secNluc that were inserted in the N/P IGR compared to those that were inserted in G/L IGR. This could potentially be due to the distance of the reporter gene from the transcriptional promoter site [33, 48]. Based on the results and previous observations, the data provided evidence that expression of reporter genes inserted in the RABV genome was influenced not only by the length of IGR sequences upstream of the gene of interest but also by the insertion distance from the promoter site.

To further ensure that the reporter gene was expressed efficiently, the start and stop signals were added in the 5’ and 3’ ends of the *NanoLuc* gene sequence, respectively. The start and stop signals are essential to guide the viral RNA-dependent RNA-polymerase to transcribe the RABV genes during transcription. The rRABVs also demonstrated stable expression of NanoLuc over ten serial passages. The lack of recombination that has been observed in the nonsegmented rhabdoviruses could contribute to the high genetic stability of the inserted reporter gene [36].

Previous studies involving rRABVs showed several advantages of the viruses for neuronal tracing, diagnostic applications or as viral vectors [34, 35, 37, 38], therefore, further studies were performed to determine whether the generated rRABVs are suitable for quantitative measurement of antiviral compounds against rRABV. For this purpose, the correlation between infected SK-N-SH cells with CVS-Nluc (N-P) and CVS-secNluc (N-P) for 16 h which represents the first release of progeny viruses [17] was analyzed. A strong, positive correlation between NanoLuc activities of CVS-Nluc (N-P) and CVS-secNluc (N-P) with amount of input virus was observed. NanoLuc activity clearly showed a 1 $\log_{10}$ difference which correlated with a 10-fold difference of input virus. These results suggested that the rRABVs encoding Nluc or secNluc reflected the rRABV infectivity. This allowed further assessment of the usefulness of the rRABVs for measurement of the antiviral activity of the nucleoside analog ribavirin, since this compound has been shown to inhibit RABV replication *in vitro* [17, 23, 50]; however, the efficacy of ribavirin has not been demonstrated in animal models or clinical studies [14, 23, 24]. Comparable dose-dependent antiviral activity of ribavirin against WT RABV and rRABVs were evidenced by NanoLuc expression derived from both CVS-Nluc (N-P) and CVS-secNluc (N-P) constructs. Antiviral activity of ribavirin was also determined by virus titration and the EC_{50} values were in the same range (12-15 μM) with the values obtained from measurements of NanoLuc activity. Taken together, these findings demonstrate that the rRABV-encoding NanoLuc system accurately represents infection by WT RABV.

RABV continuously presents a global threat despite the existence of a safe and well-tolerated vaccine and the availability of PEP. Case fatality rates approach 100% once the patient has developed neurological symptoms. Ribavirin and interferon alpha have been used to treat rabies infected-patients but have not demonstrated efficacy [11, 14, 51]. Until now, there is only one documented case of rabies that has been successfully treated with a

combinatorial therapy, namely the “Milwaukee Protocol”; however, subsequent attempts to treat rabies patients by this protocol have failed [11, 15]. Thus, novel antiviral medications against rabies are urgently needed in acute, symptomatic disease presentations.

The rRABVs encoding NanoLuc are shown to be a potential tool for cell-based compound screening assay to circumvent the practical difficulty of employing cell viability assays since RABV infection does not usually cause CPEs [27-29]. Until recently, rRABVs encoding firefly luciferase and *Gaussia* luciferase have been generated [34, 35]. Previously, rRABVs have been reported to allow monitoring of virus replication or as a viral vector rather than to measure antiviral activity of small compounds. Furthermore, the NanoLuc reporter employed in the present study has higher luciferase activity compared to firefly luciferase [43] and it has a glow-type luminescence with a signal half-life of more than 2 h. This allows for more plates to be analyzed when using a luminometer without injectors, compared with *Gaussia* luciferase that has a flash-type luminescence and requires luminometers with injectors. Based on the results, CVS-Nluc (N-P) is the most appropriate rRABV for compound screening assays. This rRABV system allowed the convenient measurement of antiviral activity since the luciferase activity measurement can be done in the same plate used for infection.

The results presented in this study demonstrate the effectiveness of rRABVs encoding Nluc and secNluc to facilitate small compound screening assay for RABV. The rRABVs are stably expressed and demonstrated a proof-of-concept in showing the activity of an antiviral compound. The rRABVs also allow rapid quantification of virus replication compared to conventional assays. Moreover, a direct comparison between rRABVs demonstrated that despite the shorter IGR sequences upstream of the reporter genes associated with higher expression levels, the relative distance of the reporter gene to the transcriptional promoter site

also further played a role in expression of reporter gene. In future studies, the rRABVs will be employed to identify small molecule compounds that inhibit RABV.

Summary

Rabies is an invariably fatal disease caused by RABV. Once infection of the CNS occurs and symptoms are developed, the case fatality rate reaches 100% despite availability of PEP. Therefore, new antiviral therapies for rabies are urgently required. Antivirals which can inhibit virus replication can be identified through screening of small chemical compounds; however, as RABV infection does not generate easily discernible CPE *in vitro*, cell viability assays may not be feasible to observe antiviral activity of small compounds against RABV. In this study, rRABVs encoding NanoLuc were generated to facilitate the screening of small compound libraries. NanoLuc expression was confirmed during virus infection to the cells and showed that the rRABVs were capable of viral replication without decrease of luciferase activity through ten serial passages. Furthermore, the rRABVs were able to quantify the antiviral activity of the nucleoside analog ribavirin against RABV *in vitro*. These findings confirm the potential of the rRABV encoding NanoLuc system to facilitate screening of small compounds to inhibit RABV infection.

Chapter II

Investigation of Anti-Rabies Virus Activity of Ribavirin-Related Compounds

Introduction

Rabies is preventable by vaccination both in animals and humans. In addition, a PEP approved by WHO that includes the administration of rabies vaccine and rabies immunoglobulin, is currently available to immediately treat an individual that is exposed to a rabid animal and should be given before the onset of rabies symptoms [26, 52]. However, rabies is invariably fatal when the virus has already reached the CNS and neurological symptoms have developed.

Despite the few reported cases of individuals surviving symptomatic rabies, including one case that was treated with the "Milwaukee Protocol" [11], it is still extremely challenging to treat symptomatic patients in the attempt to reduce rabies mortality during this stage of the disease. Ribavirin, a nucleoside analog that has a broad-spectrum antiviral activity, has been shown to inhibit RABV replication *in vitro*; however, studies have failed to demonstrate a protective efficacy *in vivo* using a mouse model and in human clinical cases [14, 52]. The other known antiviral agents, such as interferon alpha and amantadine, have also failed to demonstrate a therapeutic efficacy mouse model and clinical cases [14, 24, 52]. Recently, favipiravir (T-705), which is also a nucleoside analog with potent antiviral activity against broad-range of RNA viruses, including orthomyxoviruses [53], paramyxoviruses [54], filoviruses [55], arenaviruses [56, 57], bunyaviruses [56], flaviviruses [58], and alphaviruses

[59, 60], has been shown to inhibit RABV replication *in vitro* and provided a moderate protection from RABV infection in a mouse model of rabies [61]. The efficacy of T-705 in treatment of acute, symptomatic clinical cases of rabies remains to be evaluated.

Based on these prior studies, it is rational to consider nucleoside analog as a practicable class of chemical compounds that could conceivably be employed to inhibit RABV replication and provide a basis for treatment of rabies-associated neurological disease. Structure of nucleoside analog resembles endogenous nucleoside found in cells that are critically required for nucleic acid biosynthesis and this compound acts as a competitive inhibitor with the natural nucleosides during viral genome replication [62]. In the case of ribavirin, its mechanism of action is thought to involve: (i) inhibition of inosine-5'-monophosphate (IMP) dehydrogenase function [63-65], (ii) interference of viral genome replication by direct incorporation into the nascent viral genomes thus inducing lethal mutagenesis [66-69], and (iii) inhibition of mRNA capping [70].

Previous studies demonstrated that antiviral agents inhibiting RABV infection can be identified through an examination of compound library using either cell-based [17, 18] or cell-free systems [19]. Cell-based systems which employ RABV infection of susceptible cell lines have been shown to provide a more robust and replicable approach to identify compounds that have antiviral activity against RABV [17, 18, 50]. Therefore, a cell-based system was performed in order to identify compounds that have antiviral activity against RABV in the present study using a previously generated rRABV encoding non-secreted NanoLuc [CVS-Nluc (N-P)] [71].

Several analogs of ribavirin were tested and their *in vitro* antiviral activity against RABV was compared to that of ribavirin to provide more detailed information with regards to the structure of chemical compounds which exert more potent inhibitory effects on viral replication. In addition, one ribavirin analog namely 5-ethynyl-1-β-D-ribofuranosylimidazole-

4-carboxamide (EICAR) has been shown to possess more potent broad-spectrum antiviral activity compared to ribavirin against a number of RNA viruses, including vesicular stomatitis virus, a prototype rhabdovirus that belongs to the same viral family as RABV [65, 72, 73]. Therefore, the antiviral activity and mechanism of action of EICAR and related compounds in inhibiting RABV replication were investigated in this study.

Material and methods

Cells

Baby hamster kidney clone 13 (BHK-21 [C-13]), SK-N-SH, and NA cells were maintained in MEM supplemented with 10% FBS. Human embryonic kidney (HEK 293T) cells were maintained in high glucose Dulbecco's modified MEM (DMEM) supplemented with 10% FBS.

Viruses

Previously generated rRABV encoding NanoLuc luciferase, CVS-Nluc (N-P) [71] and WT RABV CVS were employed in this study. Virus stocks CVS-Nluc (N-P) and WT CVS were prepared on NA cells and virus titers were determined using focus forming assay.

Compound examination

EICAR, 5-ethynyl-1-β-D-ribofuranosylimidazole-4-carbonitrile (EICNR), 5-buthynyl-1-β-D-ribofuranosylimidazole-4-carboxamide (BICAR), and 4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate (mizoribine) were kindly provided by Dr. Akira Matsuda (Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan). To measure the antiviral activity of the compounds, SK-N-SH, NA, BHK, and HEK 293T cells seeded the previous day were treated with each compound for 1 h in a 2-fold serial dilution starting from 20 μM. Ribavirin (Wako) was also included as it is has been shown to inhibit RABV replication *in vitro* [17, 23] . Thereafter, the cells were infected with CVS-Nluc (N-P) at an MOI of 0.1 for SK-N-SH cells or an MOI of 1 for NA, BHK and HEK 293T cells for 24 h. The MOI was calculated as FFU/cell. Following the incubation time, the cells were submitted to luciferase assay to measure NanoLuc activity.

Luciferase assay

Whole cell lysates were employed to measure NanoLuc activity from cells infected with rRABVs encoding Nluc using Nano-Glo luciferase assay kit (Promega) and GloMax 96 microplate luminometer (Promega). The measured NanoLuc activity was expressed in RLU/sec. The inhibitory effect of the compounds was presented as relative inhibition rate. The relative inhibition rate was calculated by following formula:

$$RIR = \frac{(VC-X)}{(VC-CC)} \times 100$$

where VC (virus control) is the NanoLuc activity of DMSO-treated and infected cells, X is the NanoLuc activity of infected cells that were treated with a given concentration of a compound, and CC (cell control) is the background signal derived from DMSO-treated and uninfected cells.

Focus forming assay

Focus forming assay was performed by inoculation of CVS-Nluc (N-P) into NA cells in 24-well plates with a 10-fold dilution series of the virus in MEM. Following incubation for 1 h at 37^{o}C on a plate shaker, an overlay medium containing MEM (supplemented with 5% FBS; penicillin, 100 U/ml; streptomycin, 100 mg/ml and 0.5% methylcellulose) was added to cells. Foci of virus were detected using FITC-conjugated anti-RABV N protein antibody (Fujirebio Diagnostics, Inc.) after 3 days of incubation. The number of foci was counted to determine the virus titer in FFU/ml.

Cell viability assay

The compound cytotoxicity was analyzed using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [74]. Briefly, monolayers of cells were seeded in 96-well plates 24 h before treatment with compounds that were 2-fold serially diluted. After

24 h of incubation, 30 µl MTT solution (5 mg/ml; Sigma-Aldrich) was added to each well and incubated for 2 h at 37^{o}C. Thereafter, 150 µl of cell culture medium was removed and 150 µl formazan dissolution reagent (2-propanol with 10% Triton-X100 and 0.28% HCl) was added to each well followed by an overnight incubation at room temperature (20^{o}C). Absorbance at a 570 nm wavelength with a 630 nm reference wavelength was measured using a microplate reader (model 680; Bio-Rad). The percentage of cell viability was calculated by comparing the compound-treated and control absorbance values.

Plasmids

Plasmid expressing NanoLuc-encoding minigenome of RABV-CVS (pCAGGS-RVDI-nluc) was generated following the construction strategy of firefly luciferase expressing pCAGGS-RVDI-luc [75] which was kindly obtained from Dr. Naoto Ito (Gifu University). Briefly, a fragment containing a hammerhead ribozyme, the 5' trailer region of RABV-CVS strain, an ORF of Nluc gene, the 3' leader region of RABV-CVS strain, and a hepatitis delta virus antigenomic ribozyme were synthesized (Eurofins Genomics, Ota, Tokyo). This fragment was then inserted into linearized pCAGGS-RVDI-luc by restriction enzyme digestion (XhoI and SacI), replacing the original construct that encoded the trailer and leader sequence of RABV RC-HL strain.

Three helper plasmids: RABV N, P, and L proteins [pCXSN-N-HA(C), -P-HA(C), and -L-HA(C)] were also generated. A fragment containing the ORF of N, P, or L gene was PCR-amplified using pCVS [71] as a template and an hemagglutinin (HA) tag was added into the C-terminal end of ORF of each protein for later detection of the expressed proteins. The N and P gene fragment were inserted into pCXSN vector using XhoI and NotI enzyme restriction sites while the insertion of L gene fragment into the vector was done by an In-Fusion (Takara Bio) cloning strategy.

RABV minigenome assay

NA cells seeded in 96-well plates were treated with serially diluted EICAR, EICNR or ribavirin for 1 h and then transfected with the RABV plasmids at the following ratios: 20 ng pCAGGS-RVDI-nluc; 120 ng pCXSN-N-HA(C); 12 ng pCXSN-N-HA(C); 40 ng pCXSN-N-HA(C) using TransIT-Neural (Mirus Bio LLC, Madison, WI, USA). At 72 h post-transfection, the cells were harvested and subjected to luciferase assay.

Immunofluorescence assay

SK-N-SH cells were seeded 24 h prior in 96-well plates. Following treatment with compounds for 1 h, the cells were inoculated with CVS-Nluc (N-P). The cells were washed with PBS and incubated with fresh medium containing compounds. At 16 hpi, the cells were fixed with 10% formaldehyde solution and stained with FITC-conjugated anti-RABV N protein antibody (Fujirebio Diagnostics, Inc.) and Alexa-488-conjugated anti-mouse. The fluorescence was detected using an inverted fluorescence microscope, Olympus IX70 (Olympus, Tokyo, Japan).

Time-of-addition assays

SK-N-SH cells seeded in 96-well plates were treated with 5 μM EICAR, 5 μM EICNR or 50 μM ribavirin for 1 h at 37ºC. The culture medium was removed and the cells were washed with PBS followed by inoculation with CVS-Nluc (N-P) at an MOI of 0.1. After 1 h of incubation, the viral inoculum was removed and the cells were washed with PBS. A fresh culture medium without any compounds was feed to the cells. At 16 hpi, the cells were harvested and subjected to luciferase assay.

CVS-Nluc (N-P) was incubated with EICAR or EICNR at a final concentration of 20 μM for 1 h at 37ºC. As a control, the virus was incubated with ribavirin at a final

concentration of 50 μM. Treated virus was then used to infect confluent SK-N-SH cells seeded in 96-well plates at MOI of 0.1 with a final concentration of 0.25 μM EICAR or EICNR and 2.5 μM ribavirin which was below the EC_{50} of each compound against RABV. Following 1 h of incubation, the viral inoculum was removed and the cells were washed with PBS. A fresh culture medium containing no compounds was then added to the cells. At 16 hpi, the cells were harvested and subjected to luciferase assay.

SK-N-SH cells seeded in 96-well plates were infected with CVS-Nluc (N-P) at an MOI of 0.1. After 1 h of incubation at 37ºC, the viral inoculum was removed and replaced by fresh culture medium. At different time points post-infection (0, 2, 4, 6, and 8 h), the culture medium was replaced with media containing 5 μM EICAR, 5 μM EICNR or 50 μM ribavirin. The cells were then harvested at 16 hpi and subjected to luciferase assay.

Quantitative real-time reverse transcription PCR (qRT-PCR) assay

Total RNA was extracted from RABV-infected SK-N-SH cells using PureLink RNA Mini kit and TRIzol (Invitrogen, Carlsbad, CA). Amplification was performed using a one-step Brilliant III Ultra-Fast qRT-PCR master mix (Stratagene, La Jolla, CA) on the ABI StepOne Real-Time PCR System (Applied Biosystems, Inc. Foster City, CA) with the following thermocycling conditions: 50ºC for 10 minutes (mins) and 95ºC 3 min followed by 95ºC for 5 sec and 60ºC 10 sec for 40 cycles using two set of oligonucleotide primers and hydrolysis probes for the detection of RABV CVS N mRNA (primers: forward 5'-TCGAATGCTGTCGGTCATGT-3' and reverse 5'-CCGAAGAATTCCTCTCCCAAAT-3'; probe: 5'-FAM CAATCTCATTCACTTTGTTG MGB-3') and RABV CVS trailer for genome quantification (primers: forward 5'CGAAAGCCTGTGCATGCTAA-3' and reverse 5'TCTTGTATGATGCATCTTG-3'; probe: 5'-FAM CAGAGGTCCGGATTCAAGATCT MGB-3'). Another set of primers were also used to detect human β-actin mRNA as the

endogenous control using a Taqman Gene Expression Assay (HS01060665_g1, Applied Biosystems).

Results

Antiviral activity and cytotoxicity of EICAR, EICNR, BICAR, mizoribine and ribavirin

EICAR, EICNR, BICAR, mizoribine and ribavirin (Fig. 5) were examined for their antiviral activity against RABV using infection of a recombinant RABV, CVS-Nluc (N-P), in SK-N-SH cells. The recombinant virus is capable of expressing NanoLuc and has been previously used to demonstrate the antiviral activity of ribavirin against RABV [71] which was also used as reference compound for an RABV replication inhibitor in the current study. The antiviral activity of each tested compound was shown as a decrease of luciferase activity in the presence of the serially diluted compounds (Fig. 6A-E). The results showed that EICAR, EICNR, BICAR and ribavirin could decrease the luciferase activity in a dose-dependent manner; however, the most significant inhibition of luciferase activity was determined following treatment with EICAR (Fig. 6A). The calculated EC_{50} values for EICAR, EICNR, BICAR and ribavirin were 0.90 μM, 3.80 μM, 8.77 μM, and 18.55 μM, respectively (Table 1). There was no cytotoxicity determined in SK-N-SH cells throughout the experiments, indicating that the antiviral activity did not arise from a decrease of cell viability (Table 1).

Since EICAR and EICNR showed higher antiviral activities among all tested compounds (Fig. 6A and B), a time-course study was performed to examine the expression of NanoLuc across different time points (from 4 to 24 hpi). EICAR and EICNR were found to significantly decrease the luciferase activities in rRABV-infected SK-N-SH cells when compared to cells treated with DMSO as a control (Fig. 6F). Moreover, cytotoxicity and antiviral activities of EICAR and EICNR were also examined in other cell lines that are susceptible to RABV infection: namely, NA, BHK-21 and HEK293T cells, with ribavirin used as a positive control to determine that the anti-RABV activity of EICAR and EICNR is

not limited to SK-N-SH cells. The cytotoxicity and antiviral activities were expressed as CC_{50} and EC_{50}, respectively. As shown in Table 1, EICNR showed cytotoxicity in BHK cells (CC_{50}, 158.26 μM). There was no cytotoxicity of either EICAR or EICNR detected in SK-N-SH, NA, and HEK293T cells at the highest concentrations tested (200 μM). Ribavirin also showed no cytotoxicity in all tested cell lines at the highest concentration tested. Antiviral activity for EICAR and EICNR against RABV could be shown in NA, BHK and HEK293T cells with EC_{50} values ranging from <0.16 μM to 0.90 μM depending on the cell line.

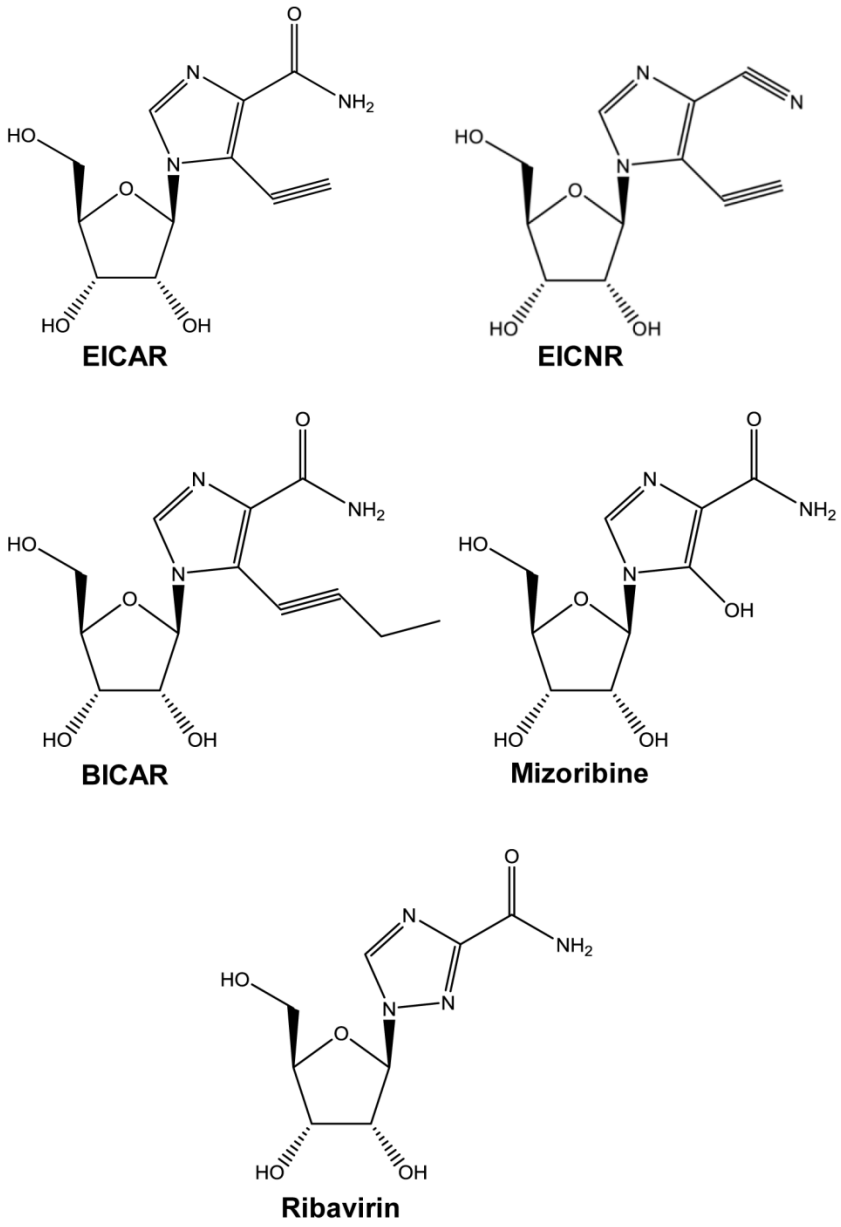

Fig. 5. Chemical structures of EICAR, EICNR, BICAR, mizoribine and ribavirin.

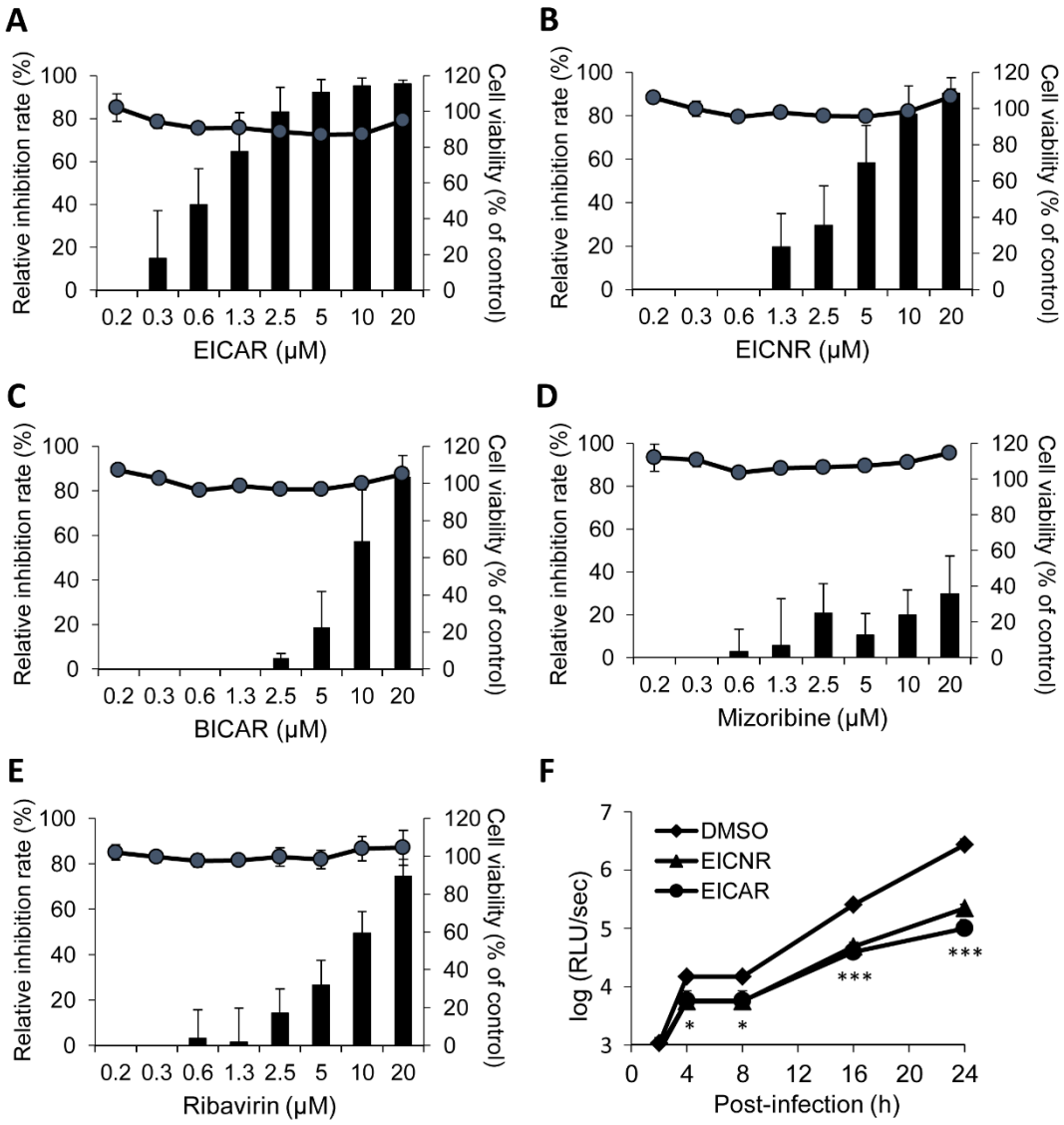

Fig. 6. Cytotoxicity and antiviral activity of compounds

(A-E) SK-N-SH cells seeded in 96-well plates were treated with EICAR (A), EICNR (B), BICAR (C), mizoribine (D) or ribavirin (E) for 1 h before infection with CVS-Nluc (N-P) at an MOI of 0.1. The infected cells were cultured in the presence of each compound for 24 hpi and then harvested for luciferase assay. Columns and solid circles represent the relative inhibition rate and the cell viability, respectively. Data represent the means and standard deviation from two independent experiments. (F) SK-N-SH cells seeded in 96-well plates were treated with 10 μM EICAR or EICNR for 1 h before infection with CVS-Nluc (N-P) at an MOI of 0.1. A luciferase assay was done in indicated time points. Data shown are representative of two independent experiments performed in triplicate. Mean and standard deviation are shown. Statistical analyses were done using Student's *t*-test (*p<0.05, ***p<0.001).

Table 1. Cytotoxicity and antiviral activity of EICAR, EICNR, and ribavirin

Cell line	Cytotoxicity [CC_{50} (μM)][a]			Antiviral activity [EC_{50} (μM)][b]		
	EICAR	**EICNR**	**ribavirin**	**EICAR**	**EICNR**	**ribavirin**
SK-N-SH	>200	>200	>200	0.90 ± 0.36	3.80 ± 1.81	18.55 ± 9.02
NA	>200	>200	>200	<0.16	0.63 ± 0.37	4.22 ± 2.13
BHK	>200	158.26 ± 41.46	>200	0.17 ±0.01	0.48 ±0.15	4.61 ±1.77
HEK293T	>200	>200	>200	0.45 ± 0.01	1.07 ± 0.01	6.62 ± 0.07

[a] The cytotoxic concentration (CC_{50}) is defined as the concentration required to reduce cell viability of a given cell line by 50%. The data represent mean values from three independent experiments.

[b] The effective concentration (EC_{50}) is defined as the concentration required to reduce NanoLuc activity of a given rRABV-infected-cell line by 50%. The data represent mean values from three independent experiments.

Confirmation of antiviral activity of EICAR and EICNR

To directly visualize the inhibition of EICAR and EICNR upon RABV infection, immunofluorescence assay was performed in SK-N-SH cells infected with CVS-WT. The infected cells were incubated with culture medium containing DMSO, 10 μM EICAR or EICNR for 16 h. No cytotoxicity observed in previous experiments at this treatment dosage. The cells were fixed and stained with antibody against RABV N to show RABV infection and Hoechst 33342 to stain cell nuclei. The representative images in Fig. 7A demonstrated the inhibition of RABV infection by EICAR and EICNR at 10 μM as less number of RABV N protein was detected when compared to infected cells that were treated with DMSO. Treatment of CVS-WT-infected SK-N-SH cells with serially diluted EICAR or EICNR also revealed that these compounds could reduce the viral titers compared to DMSO-treated and infected cells at 16 hpi (Fig. 7B and C) with higher reduction in viral titers observed in EICAR treatment.

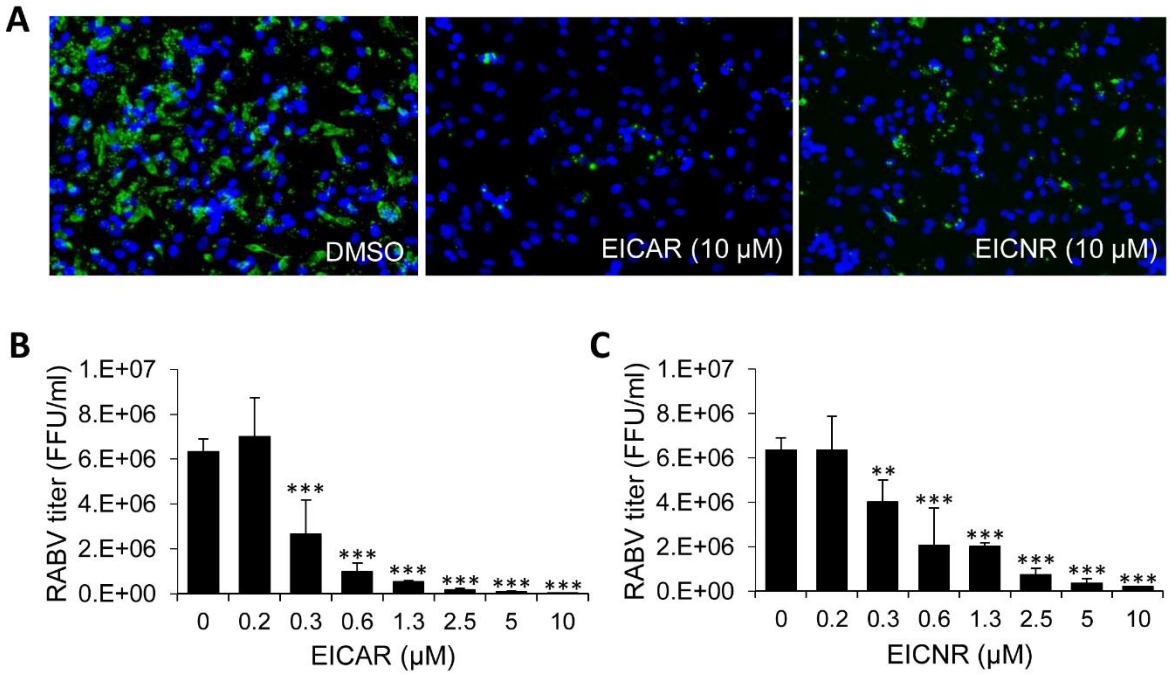

Fig. 7. Confirmation of inhibitory effect of EICAR and EICNR using immunofluorescence assay and virus titration

(A) SK-N-SH cells were infected with CVS-WT at an MOI of 5 for 1 h and treated with DMSO, 10 μM EICAR or 10 μM EICNR for 16 h. The cells were fixed with 10% formaldehyde solution before staining with FITC-conjugated monoclonal antibody directed to RABV N and Hoechst 33342 to stain cell nuclei. (B-C) SK-N-SH cells were infected with CVS-WT at an MOI 5 for 1 h and treated with serially diluted EICAR (B) or EICNR (C) for 16 h. The culture supernatants were collected and virus titers were determined by focus forming assay. Data are representative of two independent experiments performed in triplicate. Mean and standard deviation are shown. Statistical analyses were done using one way ANOVA with a Dunnett post hoc test (**p<0.01, ***p<0.001).

EICAR and EICNR inhibit the viral post-entry stage

To determine the stage of RABV life cycle that is inhibited by EICAR and EICNR treatment, time-of-addition assays were conducted. SK-N-SH cells were initially treated with DMSO, 10 μM EICAR, 10 μM EICNR or 50 μM ribavirin for 1 h before inoculation with CVS-Nluc (N-P). Fresh culture medium without any compounds was then added to the cells. At 16 hpi, the cells were subjected to luciferase assay. The results in Fig. 8A showed there was no significant difference ($p \geq 0.05$) in the luciferase activity that was observed in the cells pretreated with either EICAR, EICNR or ribavirin compared to the negative control (DMSO-treated cells) although a decrease of luciferase activity was observed in EICAR and ribavirin-treated cells.

The possibility of direct virucidal activity of EICAR and EICNR was also assessed by treating the virus stock of CVS-Nluc (N-P) with each compound for 1 h at a final concentration of 20 μM for EICAR or EICNR and 50 μM for ribavirin. The treated virus stocks were then inoculated to SK-N-SH cells followed by culturing the infected cells with a compound-free culture medium and luciferase assay was performed at 16 hpi. The results revealed that the treatment with EICAR, EICNR or ribavirin did not result in significant decreases of luciferase activities compared to the DMSO-treated control (Fig. 8B, $p \geq 0.05$).

To investigate the inhibition of RABV replication after the binding and entry of the virus into cells, an experiment was further conducted by initially inoculating CVS-Nluc (N-P) to SK-N-SH cells. Following the virus inoculation, the infected cells were treated with EICAR, EICNR or ribavirin at different time points after infection of the cells (from 0 to 8 hpi). The cells were then harvested for luciferase assay at 16 hpi. Notably, treatment with EICAR could significantly decrease luciferase activities when the cells were treated at 0-6 hpi (Fig. 8C; 0-4 hpi, $p<0.001$; 6 hpi, $p<0.01$). In contrast, EICNR only demonstrated a significant inhibitory against RABV replication when given at 0 hpi (Fig. 8D; $p<0.01$) and

ribavirin demonstrated a significant inhibition when added to the cells at 0-2 hpi (Fig. 8E; 0 hpi, $p<0.001$; 2 hpi, $p<0.01$). Altogether, these data suggested that neither EICAR nor EICNR inhibited the viral attachment or entry stage as well as these compounds do not possess direct virucidal properties against RABV. These compounds possibly act to inhibit RABV replication in the early stage of viral replication after viral attachment and entry into the cells.

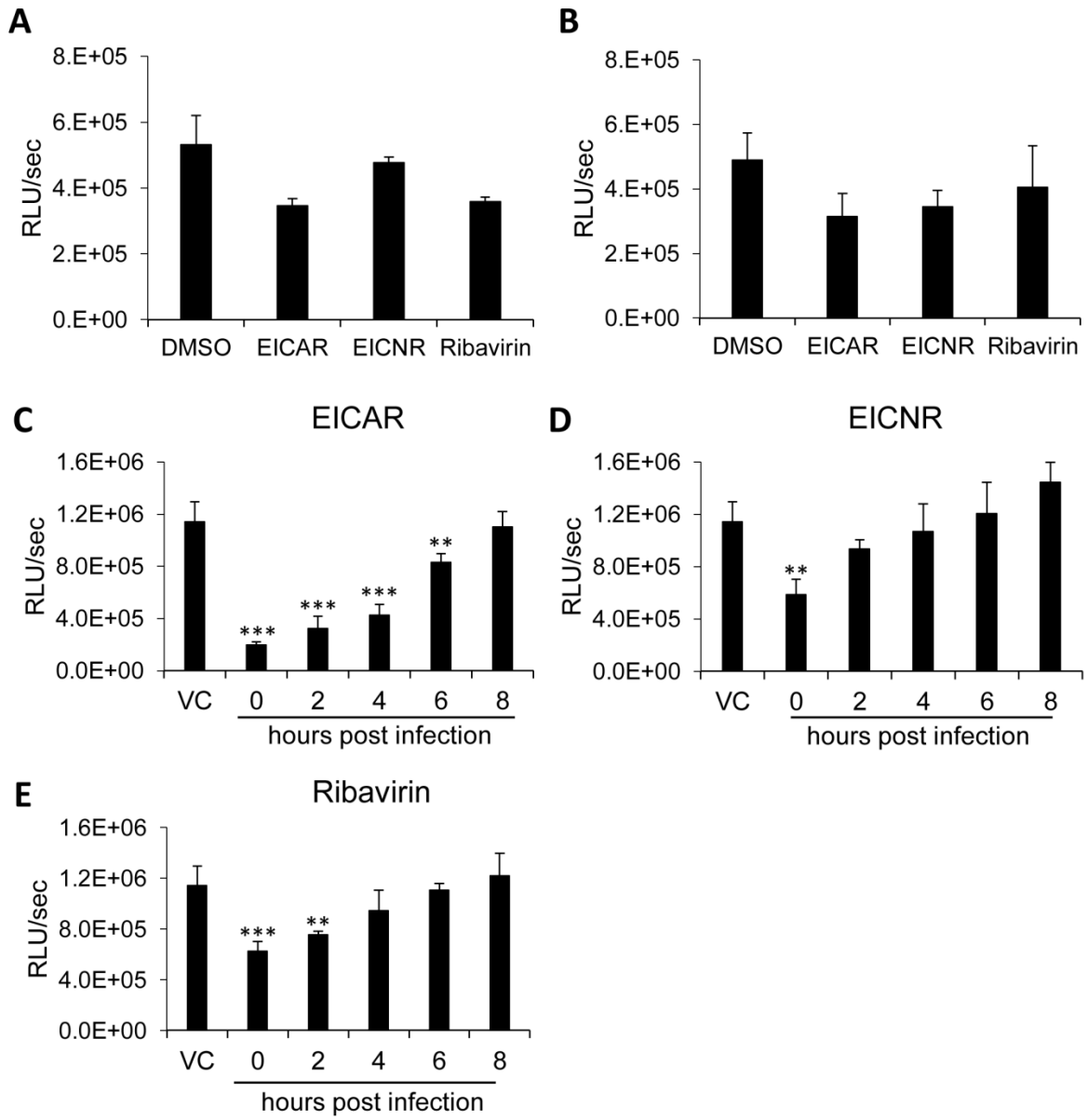

Fig. 8. Time of addition assays

(A) SK-N-SH cells were treated with DMSO, 10 μM EICAR, 10 μM EICNR or 10 μM ribavirin for 1 h before infection with CVS-Nluc (N-P) at an MOI of 0.1. Following 1 h of incubation, inocula were removed and fresh culture medium without any compounds was added to the cells. Cells were harvested and subjected to luciferase assay at 16 hpi. (B) CVS-Nluc (N-P) was incubated with DMSO, EICAR, EICNR or ribavirin at 20 μM for 1 h prior to infection to SK-N-SH cells, for a final concentration of each compound of 0.25 μM and an MOI of 0.1. Fresh culture medium without any compounds was added to the cells following 1 h inoculation with compound-treated virus. At 16 hpi, the cells were harvested and subjected to luciferase assay. (C-E) SK-N-SH cells were inoculated with CVS-Nluc (N-P) at MOI of 0.1 for 1 h. At 0-8 hpi, a final concentration of 5 μM EICAR (C), 5 μM EICNR (D) or 50 μM

ribavirin (E) was added to the cells. VC, virus control (infected-cells treated with DMSO). All presented data are representative of three independent experiments performed in triplicate. Mean and standard deviation are shown. Statistical analyses were done using one way ANOVA with a Dunnett post hoc test (**p<0.01, ***p<0.001).

EICAR and EICNR inhibit viral transcription and genome replication

The role of EICAR and EICNR in inhibiting RABV replication by targeting the post-binding and entry stage was next investigated. To determine whether EICAR and EICNR inhibit viral transcription, a RABV minigenome system was employed in the presence of these compounds and ribavirin was used at a positive control of viral transcription inhibition. An RABV CVS minigenome plasmid expressing Nluc, a non-secreted NanoLuc, was co-transfected into NA cells together with helper plasmids encoding RABV N, P, and L. The expression of NanoLuc demonstrated that all compounds possessed a dose-dependent inhibitory activity upon viral transcription as shown by the decrease of NanoLuc activity (Fig. 9). Notably, EICAR showed significant inhibitory effects on all the concentrations tested (0.2-5 µM, $p<0.001$; Fig. 9A). On the other hand, EICNR inhibited viral transcription at concentrations between 1.3-5 µM (1.3 µM, $p<0.01$; 2.5-5 µM, $p<0.001$; Fig. 9B), while inhibitory effects of ribavirin were observed in the range 6.3-50 µM (6.3 µM, $p<0.01$; 12.5-50 µM, $p<0.001$; Fig. 9C). These results indicated that EICAR and EICNR were more potent inhibitors of RABV replication than ribavirin *in vitro*.

Based on the previous results, EICAR exhibited increased antiviral activity *in vitro* compared to EICNR and ribavirin. Therefore, the ability of EICAR to inhibit viral transcription was further examined. SK-N-SH cells were infected with CVS-WT for 16 h in the presence of EICAR (1.25-5 µM). The RNAs were harvested and analyzed by qRT-PCR targeting the RABV CVS trailer and N mRNA. As shown in Fig. 10, EICAR significantly inhibited both viral replication as depicted by the decrease of N mRNA quantity (Fig. 10A, $p<0.001$) and genome replication as represented by the decrease in trailer mRNA quantity (Fig. 10B, $p<0.001$). These results suggested that antiviral activity of EICAR is attributed to its ability to interfere with the viral transcription and genome replication stages of the RABV life cycle *in vitro*.

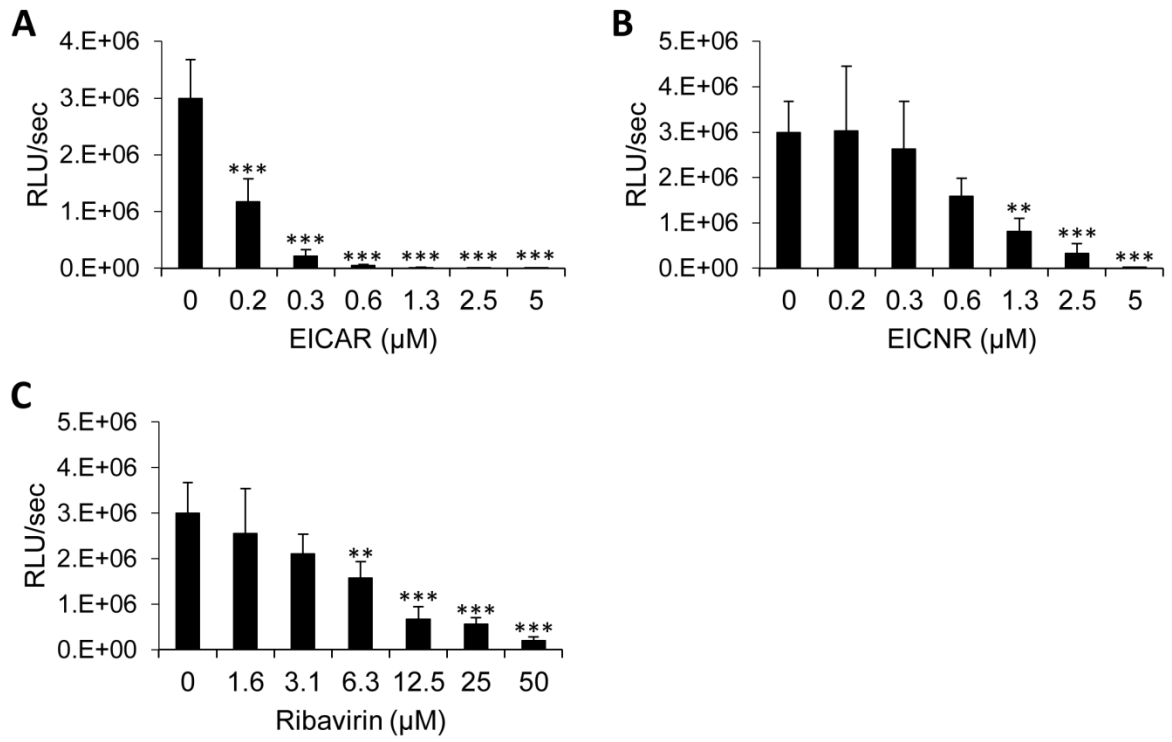

Fig. 9. Effect of EICAR, EICNR, and ribavirin treatment on minigenome-based gene expression

(A-C) SK-N-SH cells were transfected with a minigenome plasmid expressing Nluc along with helper plasmids expressing RABV polymerase complex, N, P, and L in the presence of serially diluted of EICAR (A), EICNR (B) or ribavirin (C) was added to the cell. All shown data are representative of three independent experiments performed in triplicate. Mean and standard deviation are shown. Statistical analyses were done using one way ANOVA with a Dunnett post hoc test (**p<0.01, ***p<0.001).

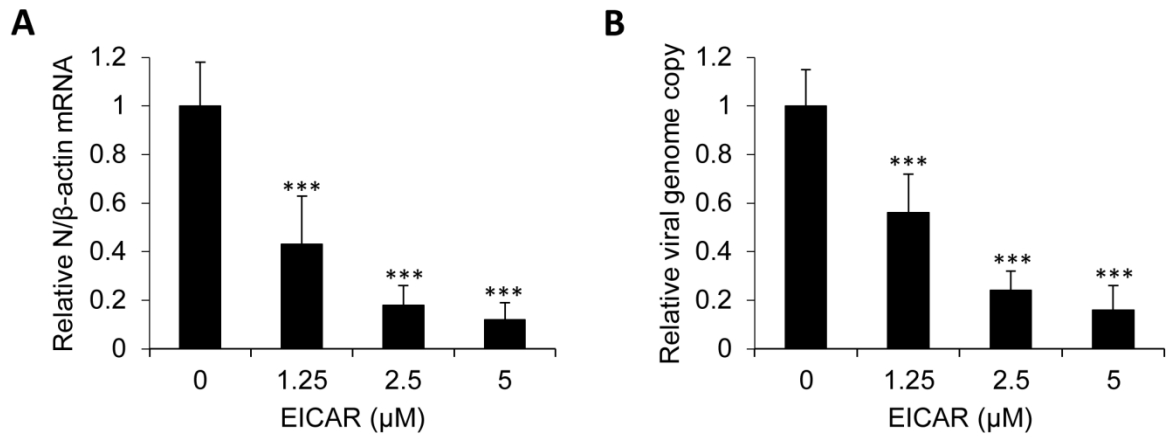

Fig. 10. Antiviral effect of EICAR treatment on viral transcription and genome replication.

(A and B) SK-N-SH cells were infected with CVS-WT at MOI 0.1 for 1 h and treated with serially diluted EICAR for 16 h. Total RNA was extracted and the RABV N mRNAs (A) and viral genome copy (B) were quantified using qRT-PCR assay and normalized to human β-actin mRNA. All shown data are representative of three independent experiments. Mean and standard deviation are shown. Statistical analyses were done using one-way ANOVA with a Dunnett post hoc test (***p<0.001).

Discussion

Rabies still represents a major threat to human health globally due to the lack of an approved effective drug that can inhibit the RABV infection once rabies symptoms develop. Therefore, there is still an urgent need to identify new antiviral agents that can reduce rabies fatality and improve survival rates. A broad-spectrum nucleoside analog, ribavirin, has been shown to demonstrate antiviral activity against RABV *in vitro*; however, when it is used to treat human cases, there was no apparent clinical efficacy observed [14, 16, 52]. Nevertheless, the use of nucleoside analogs should be taken into account when attempting to discover novel antiviral agents that inhibit RABV infection as modifications of the chemical structure of this drug class may conceivably elicit greater antiviral effects *in vitro* and *in vivo*.

In this study, several nucleoside analogs related to ribavirin (EICAR, EICNR, BICAR, and mizoribine) were examined during RABV infection *in vitro*. It was found that EICAR demonstrated an anti-RABV activity that is 20 to 27-fold more active than ribavirin while EICNR was also found to be more active than ribavirin (5 to 10-fold) although to a lesser extent than EICAR. Previous report showed that EICAR has been determined to be a potent inhibitor of cell proliferation by inhibiting cellular DNA and RNA synthesis but not protein synthesis, a phenomenon that was as also seen in ribavirin [72]. Given the similar chemical structure between EICAR and EICNR, such characteristics might also be attributable to EICNR. However, the cytotoxicity data from all tested cell lines indicates that the antiviral activities of EICAR and EICNR against RABV might not be related to their possible effect of these compounds on cellular nucleic acid metabolism.

Both EICAR and EICNR possess potency as a model of compound that inhibits RABV infection along with ribavirin and the possible target of their antiviral activity in RABV life cycle was investigated in the subsequent experiments and ribavirin was included as a

reference compound that has a known antiviral activity against RABV infection *in vitro*. Initial examination of the EICAR and EICNR antiviral target(s) revealed that these compounds likely did not act at the viral entry step nor directly inactivate the virus thus preventing viral entry into cells as when the cells were pretreated with the compounds there was no significant inhibition (Fig. 8A and B). These compounds could inhibit rRABV NanoLuc reporter gene expression when they were added to the infected cells in the early stages of viral infection post-attachment and entry of the virus into the target cells (Fig. 8C and E). Importantly, EICAR could decrease the expression of NanoLuc when the infected cells were treated until 6 hpi; thus showing that EICAR had a better antiviral activity when compared to both EICNR and ribavirin (Fig. 8C). These results suggest that EICAR and EICNR may act by interfering directly with viral transcription, possibly in a similar manner to that previously demonstrated for EICAR and ribavirin in other RNA viruses [65, 72, 73]. Nonetheless, the possibility that these compounds may inhibit the assembly and budding stage of RABV could not be eliminated as both compounds, particularly EICNR, showing more potency to reduce viral titers at a concentration below the EC_{50} value during infection with higher MOI in human neuroblastoma cells (Fig. 7B and C).

Examination using a RABV minigenome system demonstrated that EICAR and EICNR were capable of blocking viral transcription as NanoLuc expression was decreased in a dose-dependent manner. Similar observation was also found following ribavirin administration. It is plausible that EICAR and EICNR possess a similar target as ribavirin in the inhibition of viral transcription [23, 66] in RABV infection as both compounds are analogs of ribavirin. Furthermore, RABV N mRNA synthesis and genome replication, as represented by the quantity of RABV trailer mRNA, were inhibited by EICAR. Taken together, these results highlight the possible role of EICAR in inhibiting RABV replication during the stages of both viral transcription and genome replication.

Previous studies have investigated possible mechanisms by which EICAR demonstrates its antiviral activity. EICAR can act as an IMP dehydrogenase inhibitor by a similar mechanism of action to that was proposed for ribavirin [65, 73]. Inside the cell, EICAR is metabolized to EICAR 5'-monophosphate which shares similar binding site on IMP dehydrogenase to IMP [64]. Inhibition of IMP dehydrogenase leads to a reduction in the level of guanine nucleotide pool. Evidence in support of this proposed mechanism has been provided by reversing the antiviral activity of EICAR and ribavirin with addition of exogenous guanosine [63-65, 76, 77].

The marked difference between EICAR and ribavirin lies on the substitution of the N at position 2 of the triazole ring with an alkynyl-carbon moiety, while EICNR is the 4-cyano derivative of EICAR (Fig. 5). This structural difference might contribute to the enhancement of the antiviral activity of EICAR and EICNR compared to ribavirin; however, further structure-activity relationship study needs to be performed to elucidate the role of the compounds structure to inhibit RABV replication.

These findings suggest that EICAR is a more potent anti-RABV compound compared to ribavirin by inhibiting viral transcription and genome replication. Further works are required to determine the precise mechanism of action of the EICAR inhibitory effect upon RABV replication *in vitro* and *in vivo* studies using models of RABV infection remain to be explored to determine the efficacy of EICAR in infected animals.

Summary

Nucleoside analogs, such as ribavirin, can inhibit RABV replication *in vitro* and serve as a potent class of antiviral compounds for the development of novel antiviral agents against RABV. In this study, several other nucleoside analogs harboring similar chemical structures with ribavirin were examined in the attempt to identify potential compounds with comparable or superior antiviral activity to ribavirin. Employing the cell infection system with previously generated rRABV encoding NanoLuc, it was shown that two compounds (EICAR and EICNR) possess more potent antiviral activity against RABV when compared to ribavirin with antiviral activity of EICAR was in the submicromolar concentration range. Moreover, there is no discernible cytotoxicity observed in the effective concentrations employed in human neuroblastoma, mouse neuroblastoma, baby hamster kidney, and human embryonic kidney cells. These compounds could inhibit RABV replication in a dose-dependent manner and similar antiviral activities were also observed in different cell lines infected with RABV.

Subsequent investigations revealed that EICAR and EICNR likely do not inhibit RABV cellular attachment and entry nor inactivate the virus directly. However, both EICAR and EICNR demonstrated an inhibition of viral gene expression as represented by the decrease of NanoLuc activities expressed by the rRABV used in the studies when these compounds were added into the cells in the early stage of viral replication at the post-entry stage of the viral life cycle. Moreover, EICAR showed higher inhibitory activity when compared to EICNR and ribavirin. Further study with a RABV minigenome assay, which reflected the viral transcription and genome replication stage in the RABV life cycle, demonstrated that the inhibitory activity of EICAR and EICNR is attributable to their ability to interfere with viral transcription. A similar phenomenon was also observed using qRT-PCR which confirmed that EICAR, the most potent antiviral agent against RABV in this study, act by inhibiting the

viral transcription and genome replication stages. Taken together, these findings correspond to the possible mechanism action in which nucleoside analog inhibits viral replication by interfering with viral transcription and genome replication.

Conclusions

Despite the availability of immunoprophylaxis treatment against rabies that serves both as preventive and therapeutic measures, there is no currently available medication that provides effective treatments for RABV-infected patients and almost 100% of the symptomatic cases are fatal. Therefore, novel antiviral agents that can inhibit RABV replication are still urgently needed and such agents can be discovered through examination of compound libraries.

In chapter I, rRABVs encoding NanoLuc were generated to facilitate the screening of compound libraries for viral inhibitors. The infectivity of rRABVs was determined by measurement of viral titres and quantification of NanoLuc expression after inoculation of rRABV into cells. Detailed characterization of the rRABV showed that the luciferase activities could be maintained over ten serial passages of the rRABV and correlated with the viral inputs. In addition, the rRABV could be used to demonstrate a dose-dependent antiviral activity of ribavirin against RABV, reflected by the decrease of NanoLuc signal.

In chapter II, the rRABV encoding Nluc, non-secreted type NanoLuc, was employed to examine the antiviral activity of ribavirin-related compounds. It was found that EICAR, EICNR, and BICAR could decrease the Nluc expression in the rRABV-infected human neuroblastoma cells with more than 5 to 27- fold higher activities compared to ribavirin in a dose-dependent manner and superior antiviral activity was observed in EICAR and EICNR treatment compared to BICAR and RBV ribavirin treatment of RABV-infected cells. The antiviral activity of EICAR and EICNR against RABV replication was then examined in a time-of-addition assay which suggests that EICAR inhibits the viral transcription and genome replication stages of the RABV life cycle. These findings were confirmed by use of a RABV minigenome assay and qRT-PCR quantification of viral gene expression and genome copy.

The findings presented in this thesis provide insights into screening methodologies and chemical compounds that could feasibly be employed to generate antivirals to inhibit RABV infection in exposed individuals in order to decrease the morbidity and mortality associated with rabies.

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