



# Antiviral effect of sinefungin on in vitro growth of feline herpesvirus type 1

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Received: 19 March 2019 / Revised: 24 August 2019 / Accepted: 27 August 2019 / Published online: 18 September 2019  
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## Abstract

Feline herpesvirus type 1 (FHV-1) causes a potentially fatal disease in cats. Through the use of virus inhibition and cytotoxicity assays, sinefungin, a nucleoside antibiotic, was assessed for its potential to inhibit the growth of FHV-1. Sinefungin inhibited in vitro growth of FHV-1 most significantly over other animal viruses, such as feline infectious peritonitis virus, equine herpesvirus, pseudorabies virus and feline calicivirus. Our results revealed that sinefungin specifically suppressed the replication of FHV-1 after its adsorption to the host feline kidney cells in a dose-dependent manner without obvious cytotoxicity to the host cells. This antibiotic can potentially offer a highly effective treatment for animals infected with FHV-1, providing alternative medication to currently available antiviral therapies.

## Introduction

Nucleoside antibiotics constitute a large family of important microbial products with antiviral, antifungal and anti-protozoal activities [1]. Because nucleosides metabolites play pleiotropic roles, such as energy donors, cofactors and metabolite carriers, in most primary metabolisms and central roles in genetic inheritance, they possess high potential of targeting parasite-specific process, such as viral proliferation and protozoan parasitism [2].

Sinefungin is a nucleoside antibiotic and structural analogue of *S*-adenosyl-L-methionine, and it inhibits the methylation of DNA, RNA, proteins and other molecules [3–6]. It has been suggested that the potent antiviral activity of sinefungin might be due to selective inhibition of cap-methylation in maturing mRNA molecules, particularly by RNA (guanine-N7) methyltransferase, which adds a methyl group to Gppp-RNA for the formation of the m7GpppRNA cap. This process is essential for the initiation of translation

and protection of RNA molecules from degradation by 5'-exonucleases [7–10].

In this study, we assessed the antiviral activity of sinefungin on some major animal viruses. Feline herpesvirus type 1 (FHV-1) is an enveloped DNA virus, classified into the family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus*. This virus causes viral rhinotracheitis in cats. This commonly diagnosed clinical disease is characterised by upper respiratory symptoms and conjunctivitis [11–14]. Although most kittens can recover from primary infection with FHV-1, the virus can latently infect the trigeminal ganglion, often becoming reactivated in old and/or immunocompromised cats. When FHV-1 infects newborn, debilitated and immunodeficient cats, secondary infections with other pathogens occur and sometimes the disease becomes fatal.

In addition to FHV-1, we evaluated some other viruses which can infect animals. Feline infectious peritonitis virus (FIPV) is a member of the family *Coronaviridae*, subfamily *Coronavirinae*, genus *Alphacoronavirus*; it has a single-stranded positive-sense RNA genome. FIPV is a causative agent of feline infectious peritonitis, which is a systemic and fatal immune-mediated disease [15]. Equine herpesvirus type 1 (EHV-1) and Pseudorabies virus (PRV) belong to the family *Herpesviridae*, genus *Varicellovirus*; they possess double-stranded DNA genome similar to FHV-1. EHV-1 causes severe respiratory disease, neurological disease, abortion or death in horses [16, 17]. PRV causes Aujeszky's disease, a neurological and respiratory disease

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in pigs and a lethal disease in other animals [18]. Feline calicivirus (FCV), which contains a single-stranded positive-sense RNA genome, belongs to the family *Caliciviridae*, genus *Vesivirus*; it causes acute oral and upper respiratory disease in cats [19].

Here we report the *in vitro* antiviral effect of sinefungin on the growth of FHV-1, FIPV, EHV-1, PRV and FCV. In addition to its antiviral activity, the cytotoxicity of sinefungin toward host cells was assessed to determine its potential as an antiviral treatment for infected animals.

## Materials and methods

### Cells and viruses

A feline kidney cell line, CRFK [20], Fcwf-4 cell line [21] and our established fetal horse kidney (FHK)-Tcl3.1 cell line [22] were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Carlsbad, CA, USA) containing 10% heat-inactivated fetal calf serum (FCS) (GE Healthcare Life Science, UK), 100 U ml<sup>-1</sup> of penicillin and 100 µg ml<sup>-1</sup> of streptomycin (Thermo Fisher Science, USA) in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. FHV-1 C7301 strain [17], FCV F9 strain [23], FIPV M91-267 strain [24], PRV Indiana strain [25] and EHV-1 89c25p strain [26] were used in this study. FHV-1, FCV and PRV were propagated in CRFK cells, FIPV was propagated in Fcwf-4 cells [27] and EHV-1 in FHK-Tcl3.1 cells cultured in DMEM with 2% FCS at 37 °C in 5% CO<sub>2</sub> until cytopathic effect was observed and then were stored at -80 °C. Sinefungin was purchased from Sigma-Aldrich (MO, USA), and the purity of >98% was confirmed by our analytical HPLC system implemented with Hitachi photodiode-array detector and a CAPCELL PAK SCX column (Shiseido, Japan) as previously described [28].

### Virus inhibition assay

Host cell lines, CRFK, Fcwf-4, and FHK-Tcl3.1, were cultured at 37 °C on 12-well culture plates (Sumitomo Bakelite, Tokyo, Japan) in DMEM containing 10% FCS. Next, the medium was removed and cells in each well were inoculated with FHV-1, FCV, FIPV, PRV or EHV-1 at a multiplicity of infection (M.O.I.) of 0.01 in 200 µl of DMEM containing 2% FCS with/without sinefungin (100 µg ml<sup>-1</sup>; 262 µM). After incubation for 1 h at 37 °C, cells were washed twice with DMEM and overlaid with media containing 2% FCS with/without sinefungin (100 µg ml<sup>-1</sup>). After inoculation at 37 °C, FCV was collected at 48 h post-infection (h.p.i.), FIPV at 36 h.p.i. and FHV-1, PRV and EHV-1 at 72 h.p.i. The supernatant was collected and stored at -80 °C, and virus titres were determined by plaque assay. The percentage of plaque number

was calculated in comparison with that of the untreated control. Each experiment was repeated at least twice.

### EC<sub>50</sub> determination

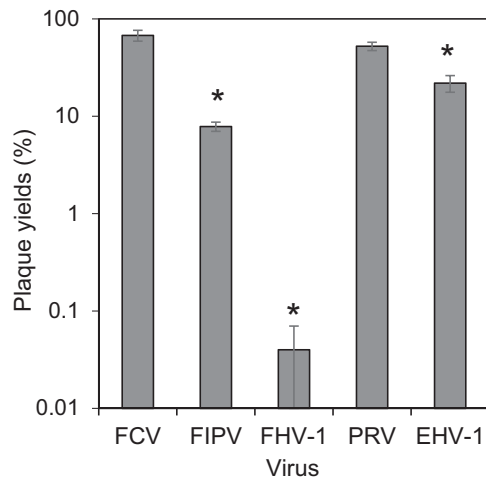
CRFK cells were cultured on 12-well culture plates in DMEM containing 10% FCS in a humidified atmosphere with 5% CO<sub>2</sub> at 37 °C. Next, the media was removed and cells in each well were inoculated for 1 h with FHV-1 at an M.O.I. of 0.01 in 200 µl of DMEM containing 2% FCS with/without twofold diluted sinefungin or acyclovir (3–100 µg ml<sup>-1</sup>) at 37 °C or without sinefungin at 4 °C. The cells were then washed twice with DMEM. After washing, the cells were overlaid with media containing 2% FCS with/without the antiviral agent (3–100 µg ml<sup>-1</sup>) and incubated at 37 °C. After incubation, the supernatant was collected and stored at -80 °C until virus titration assay. Each experiment was repeated at least twice.

### Virus titration

Cell lines, CRFK or FHK-Tcl3.1, cultured on six-well plates (Sumitomo Bakelite, Tokyo, Japan), were inoculated with 200 µl of tenfold diluted viral solutions and incubated at 37 °C for 1 h. After incubation, the media were removed, the cells were washed twice with DMEM and then overlaid with 0.8% agarose (SeaPlaque GTG Agarose; Lonza, Basel, Switzerland) in DMEM containing 6.7% FCS. The plates were incubated at 37 °C until plaques were observed. Next, the cells were fixed with phosphate-buffered formalin and stained with crystal violet to count the number of plaques. The percentage of plaque numbers was calculated by comparison with that of the untreated control.

### Cytotoxicity assay

The cytotoxicity of sinefungin was assessed using MTT assay as described in a previous report [29]. CRFK cells were inoculated at a density of 10<sup>4</sup> cells/well in 96-well plates. After 24 h, the cells were incubated with different concentrations (3–100 µg ml<sup>-1</sup>) of sinefungin. Following incubation for 72 h, the cells were washed once with phosphate-buffered saline and 100 µl of fresh DMEM was added to the wells. Subsequently, 10 µl of MTT reagent was added to each well and the plates were incubated for 4 h at 37 °C. Following incubation, 100 µl of 10% sodium dodecyl sulphate in 1 mM HCl was added to each well and the plates were incubated for 4 h at 37 °C. The optical density was measured at 570 nm using a spectrophotometer (Bio-Rad, Tokyo) and the percentage cell viability was calculated in comparison with that of the untreated control after subtracting the background. Each experiment was repeated at least twice.



**Fig. 1** Antiviral effects of sinefungin toward FCV, FIPV, FHV-1, PRV and EHV-1. CRFK cells were propagated with FCV, FIPV, FHV-1 or PRV and FHK-Tcl3.1 cells were propagated with EHV-1 at an M.O.I. of 0.01 with/without sinefungin ( $100 \mu\text{g ml}^{-1}$ ;  $262 \mu\text{M}$ ).  $*p < 0.0001$  referenced to the plaque yield by FCV

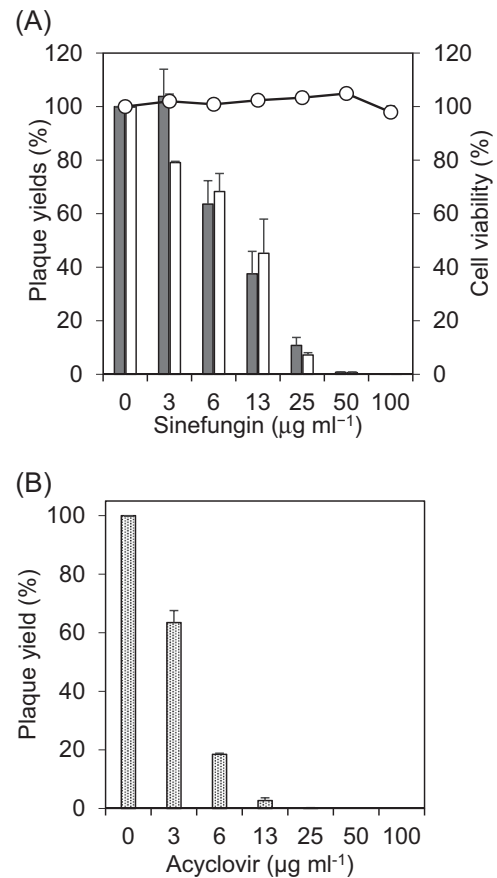
## Results

### Antiviral effects of sinefungin

A variety of viruses were used to assess the antiviral effects of sinefungin. CRFK cells were inoculated with FCV, FIPV, FHV-1, or PRV, and FHK-Tcl3.1 cells were inoculated with EHV-1. After incubation, the supernatants were collected, and the virus titre was determined by plaque assay. Figure 1 presents the percentage of plaque number standardised with plaque number of the untreated control. The plaque numbers for FCV and FIPV decreased to 67.7% and 7.8%, respectively. Thus, sinefungin was most effective against FHV-1 infection (0.04%). Alternatively, PRV and EHV-1, which belong to the genus *Varicellovirus* similar to FHV-1, were less sensitive to sinefungin, and the plaque yields were 52.4% and 21.9%, respectively. The antiviral activity of sinefungin was reproduced at the similar levels on another FHV-1 isolate, Tokyo/181128, and when the host was Fcwf-4 cell (data not shown).

### EC<sub>50</sub> of sinefungin against FHV-1 infection

To investigate whether sinefungin inhibits viral adsorption to host cells, CRFK cells were incubated with FHV-1 and with/without different concentrations of sinefungin ( $3\text{--}100 \mu\text{g ml}^{-1}$ ) at 37 or 4 °C for 1 h and washed with DMEM. After incubation for 72 h with/without sinefungin, the viruses were collected, and the titres were determined. The result showed that sinefungin specifically suppressed FHV-1 propagation in CRFK cells in a dose-dependent manner. The EC<sub>50</sub> values at each incubation temperature were nearly identical (EC<sub>50</sub> at 37 °C:  $9.5 \mu\text{g ml}^{-1}$ , at 4 °C:  $11.2 \mu\text{g ml}^{-1}$ )



**Fig. 2 a** Dose-dependent anti-FHV-1 activity of sinefungin and its cytotoxicity in CRFK cells. CRFK cells were inoculated with FHV-1 at an M.O.I. of 0.01 with different concentrations of sinefungin ( $0\text{--}100 \mu\text{g ml}^{-1}$ ) at 37 °C (dark bar) or at 4 °C (white bar) for 1 h. After adsorption, the cells were incubated in the presence of twofold diluted sinefungin solution ( $0\text{--}100 \mu\text{g ml}^{-1}$ ) at 37 °C and the virus titre was determined by plaque assay. The percentage of viable cells is represented by the white circle. **b** Dose-dependent anti-FHV-1 activity of acyclovir in CRFK cells. The antiviral effect was tested under the same condition with different concentrations of acyclovir ( $0\text{--}100 \mu\text{g ml}^{-1}$ ) at 37 °C (meshed bar)

(Fig. 2a). Sinefungin may not affect the attachment of FHV-1 to the host cells, but it may affect the biochemical processes after adsorption. The EC<sub>50</sub> value of acyclovir ( $18 \mu\text{g ml}^{-1}$ ) was determined by our in vitro assay (Fig. 2b), suggesting that sinefungin is as effective as the anti-herpes drug, acyclovir, which is used for the treatment of shingles.

### Cytotoxicity of sinefungin toward CRFK cells

The cytotoxicity of sinefungin toward CRFK cells was assessed using an MTT assay (Fig. 2a). There was no significant difference in cell viability at concentrations of  $3\text{--}100 \mu\text{g ml}^{-1}$ , which suggested that sinefungin has almost no cytotoxicity toward CRFK cells at effective antiviral concentrations.

## Discussion

The antiviral activity of sinefungin has been reported to be because of the potent inhibition of mRNA-methyltransferase and multiplication of the vaccinia virus [30] or inhibition of cell transformation by the Epstein–Barr virus [31]. In this study, we assessed the antiviral effects of sinefungin on some major animal viruses. The results indicate that FHV-1 is more sensitive to sinefungin than other cat and herpes viruses. The mechanism underlying inhibition remains unclear; however, this antiviral effect appears not specific to the genus *Varicellovirus* because FIPV and EHV-1 were also inhibited by sinefungin, while PRV and FCV were less sensitive to sinefungin in CRFK (Fig. 1a). It is unknown whether the mechanism of action of sinefungin against FHV-1 is due to its activity as an inhibitor against *S*-adenosyl-L-methionine-dependent methyltransferase reactions [3–6].

Further investigations were conducted to determine the antiviral activity of sinefungin against FHV-1 in detail. Sinefungin inhibited FHV-1 replication in a dose-dependent manner with an EC<sub>50</sub> value of 25 μM (9.5 μg ml<sup>-1</sup>) at 37 °C. Few reports on the comparison of antiviral drugs against FHV-1 are available. For example, acyclovir is typically used for the treatment of an ocular disease that is caused by FHV-1 infection with an EC<sub>50</sub> of 250 μM. Adefovir inhibits herpesvirus infection, including those caused by human simplex virus (HSV) [32], cytomegalovirus (CMV) [33] and varicella-zoster virus [34]; it can inhibit FHV-1 infection in vitro with an EC<sub>50</sub> value of 73 μM. Foscarnet, which is administered for CMV in human retinitis or HSV infection [32], is also effective against FHV-1 infection with an EC<sub>50</sub> value of 140 μM [35]. Accordingly, sinefungin might be as effective and useful for the treatment of FHV-1 infection as acyclovir, adefovir and foscarnet, based on the comparable EC<sub>50</sub> values revealed in our in vitro assay, and sinefungin shows no obvious cytotoxicity toward the host feline kidney cell line. A previous study reported that sinefungin was cytotoxic to NCTC clone 929 mouse cells of strain L, known as fibroblasts [30]. This toxicity might be due in part to the difference of the host and tissue from which the culture cells were derived. According to a recent database, sinefungin and acyclovir have similarly low toxicity; for sinefungin oral LD<sub>50</sub> is 1 g/kg (mouse) and subcutaneous LD<sub>50</sub> is 185 mg/kg (mouse) [36]. For acyclovir, oral LD<sub>50</sub> is >10 mg/kg (mouse) and subcutaneous LD<sub>50</sub> is 1,118 mg/kg (mouse) [37].

**Acknowledgements** This study was supported by grants from Japan Agency for Medical Research and Development (16fk0108117h0701) and by JSPS KAKENHI Grant Number 15H04599.

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

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