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Antiviral effects of simeprevir on multiple viruses

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ABSTRACT

Simeprevir was developed as a small molecular drug targeting the NS3/4A protease of hepatitis C virus (HCV). Unexpectedly, our current work discovered that Simeprevir effectively promoted the transcription of IFN-β and ISG15, inhibited the infection of host cells by multiple viruses including Zika virus (ZIKV), Enterovirus A71 (EV-A71), as well as herpes simplex virus type 1 (HSV-1). However, the inhibitory effects of Simeprevir on ZIKV, EV-A71 and HSV-1 were independent from IFN-β and ISG15. This study thus demonstrates that the application of Simeprevir can be extended to other viruses besides HCV.

Treatment of hepatitis C virus (HCV) infection has been significantly improved with the development of direct-acting antiviral agents (DAAs). Simeprevir, developed as an inhibitor for HCV (initially termed as TMC435) targeting the NS3/4A protease (Tsantrizos, 2009; Zhang, 2016), is such a DAA that has been proved very effective in treating HCV infection either alone or in combination with other drugs (Babatin et al., 2018; Gane et al., 2017). With a single oral administration, Simeprevir is extensively distributed to the liver and intestinal tract with a biovailability of 44% (Zhang, 2016). Although clinical observations implied some side effects when applied to HCV infected patients (Izzo et al., 2016; Sarkar et al., 2015), off-targeting of Simeprevir to host or other microbial proteins has not been reported yet.

In response to viral infections, mammalian hosts mount remarkable defense reactions mainly executed by the innate and adaptive immune systems. Innate immunity is considered as the first line of immune defense, which can be initiated very quickly in response to viral infection. Viral infected host cells can produce cytokines including type I interferon (IFN), which protects host through interfering viral replication via interferon stimulated genes (ISGs) such as ISG15 (Parkin and Cohen, 2001).

Zika virus (ZIKV) belongs to the family *flaviviridae*, genus *flavivirus*, which was first isolated in 1947 from a sentinel monkey in the Zika forest in Uganda, east Africa. Like other flaviviruses such as dengue, yellow fever, Japanese encephalitis and West Nile viruses, ZIKV is enveloped and icosahedral, and has a nonsegmented, single-stranded, 10-kilobase, positive-sense RNA genome (Sirohi and Kuhn, 2017). ZIKV infection causes neurological complications, microcephaly in fetus, and Guillain-Barré syndrome in adults. ZIKV could trigger type I IFN response through MAVS (IPS-1, VISA, Cardif) (Piret et al., 2018). There is no specific vaccine and antiviral drugs for Zika virus yet.

Enterovirus A71 (EV-A71) is a virus of the *enterovirus* genus in *picornaviridae* family (Lin et al., 2002; Weng et al., 2005). Like poliovirus, EV-A71 has a positive single-stranded RNA genome of about 7400 nucleotides which contains a single open reading frame (ORF) flanked by conserved and untranslated regions at both 5′ and 3′ ends. It was firstly isolated and characterized from cases of neurological disease in California in 1969 (Wang et al., 2002). EV-A71 infection causes mild hand, foot and mouth disease (HFMD), severe neurological complications, as well as deaths in infants and young children. Some studies have shown that EV-A71 actives type I interferon production, and EV-A71 can escape immune defenses by cleaving critical innate immune molecules (Kuo et al., 2013; Lei et al., 2010; Pathinayake et al., 2015). Effective antiviral agents and vaccines against EV-A71 are not available yet.

Herpes simplex virus type 1 (HSV-1) belongs to DNA virus of *alpha herpesvirus* subfamily, is a relatively large enveloped virus with a 152-kb linear double-stranded genome that codes for about 90 RNA transcripts, of which 84 appear to encode proteins. HSV-1 induces a wide variety of illnesses, including mucocutaneous infections, central nervous system (CNS) infections, and occasionally infections of the visceral organs. Although HSV-1 is in possession of several mechanisms to evade

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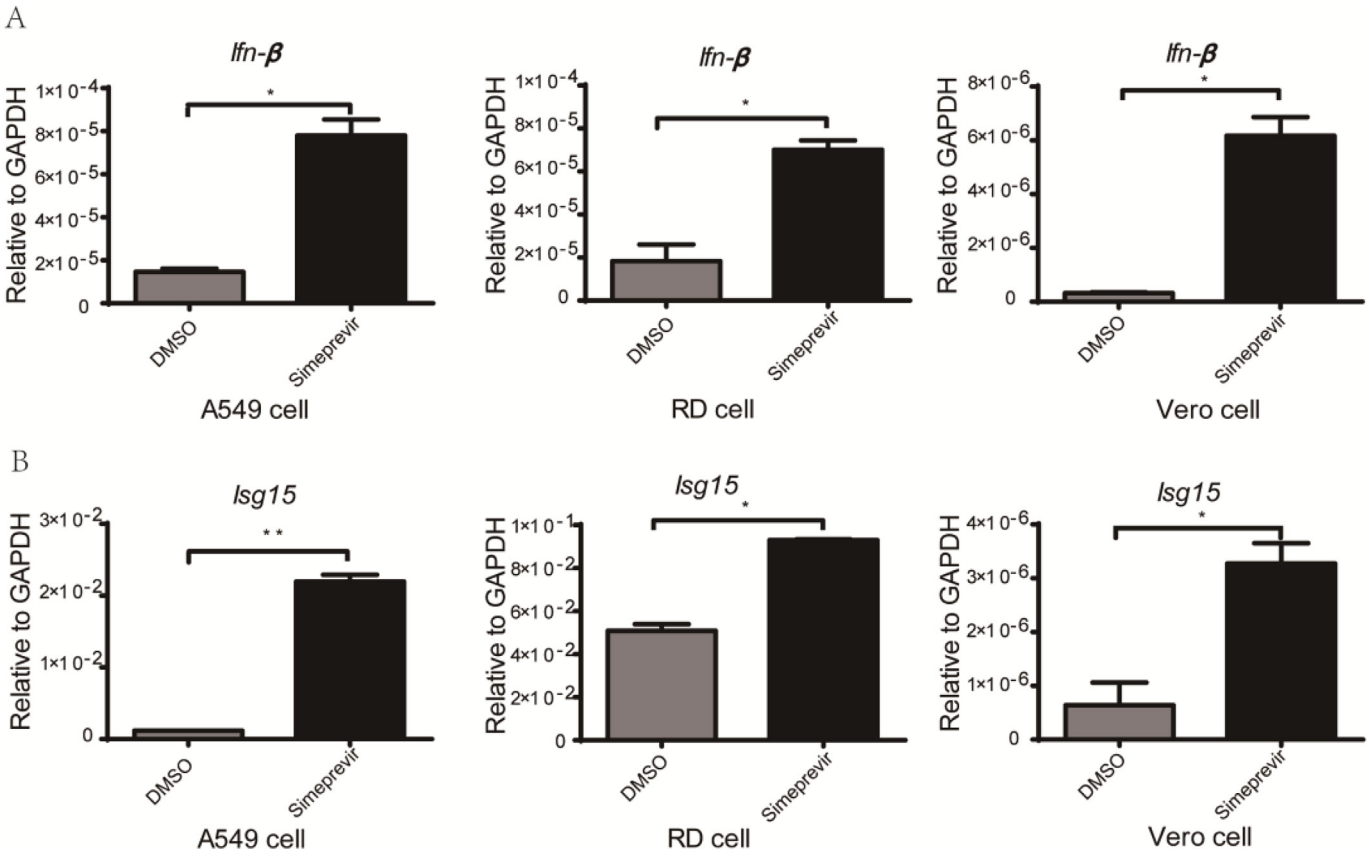


Fig. 1. Simeprevir induced the expression of *Ifn-β* and *Isg15*. A549, RD or Vero cells were treated with 5 μM Simeprevir for 24 h, then RNA was extracted for the examination of *Ifn-β* (A) *and Isg15* (B) expression through real-time PCR. The statistical differences were analyzed by t tests (and nonparametric tests). *: $P < 0.05$, **: $P < 0.01$.

IFN responses (Leib, 2002), type I IFN is known to block HSV-1 replication at an early step in replication (Mittnacht et al., 1988), which is very important for resistance against this virus (Dupuis et al., 2003; Leib et al., 1999).

Given that Simeprevir is an inhibitor for HCV targeting the NS3/4A protease (Tsantrizos, 2009; Zhang, 2016), which cleave critical innate immune adaptor molecules such as MAVS and TRIF to interfere with host anti-viral signaling (Li et al., 2005a, 2005b), we hypothesized that Simeprevir may also affect innate anti-viral signals. When A549 cells were treated with 5 μM Simeprevir for 24 h, the mRNA levels of IFN-β (Fig. 1A), as well as ISG15 (Fig. 1B), were up-regulated significantly. Besides A549 cells, we also found those effects on other types of cells such as RD cells and Vero cells, wherein Simeprevir promoted IFN-β and ISG15 transcription compared with DMSO treatment (Fig. 1 A, B).

As IFN-β and ISG15 play critical roles in limiting the spread of viral infections, we reasoned that Simeprevir may have general antiviral effects on different viruses. Both Zika virus (ZIKV) and HCV belong to *flaviviridae* family, so we tested if Simeprevir have any inhibitory effects on ZIKV infection. Of note, the transcription of ZIKV in Vero cells was clearly inhibited by Simeprevir in a dose-dependent manner (Fig. 2A). More experiments were further performed to test the antiviral effects of Simeprevir on other viruses. For RNA virus, real-time PCR results showed that Simeprevir also effectively inhibited the replication of Enterovirus A71 (EV-A71) (Fig. 2A), but not Influenza A virus (IAV) or Vesicular stomatitis virus (VSV) (data not shown). For DNA virus, Simeprevir inhibited the transcription of Herpes simplex virus 1(HSV-1) (Fig. 2A), but not Human Cytomegalovirus (HCMV), Vaccinia virus (VACV) or Murine Cytomegalovirus (MCMV) (data not shown). Moreover, the inhibitory effects of Simeprevir on ZIKV, EV-A71 and HSV-1 were dose- and time-dependent (Fig. 2A and B). In addition, intracellular EV-A71 and HSV-1 viruses were also determined by TCID50 method, which showed that EV-A71 and HSV-1 viral titers were robustly reduced after Simeprevir treatment, and the inhibitory effects were time-dependent (Fig. 2C), which was positively correlated with RNA-based experimental results (Fig. 2 B). Moreover, the antiviral effects of Simeprevir on ZIKV and HSV-1 were not only evident in Vero cells, but also reproduced in A549 cells; and that on EV-A71 were not only observed in RD cells, but also confirmed in A549 cells (Fig. 2D). Taken together, Simeprevir inhibited the propagation of ZIKV, EV-A71 and HSV-1 in various cell culture systems.

In order to explore whether IFN-β mediated the antiviral effects of Simeprevir, we interrupted the IFN-β signaling pathway through deleting the IFN-α/β receptor (IFNAR) using the CRISP/Cas9 system. Deletion of IFNAR blocked IFN-β pathway completely: although recombinant IFN-α induced robust ISG15 transcription in regular A549 or RD cells, little ISG15 mRNA was detected in cells deficient for IFNAR (Fig. 3A). However, Simeprevir treatment in A549 cells either expressing or deficient for IFNAR similarly inhibited the viral mRNA copy of ZIKV, EV-A71 and HSV-1 (Fig. 3B). In addition, in RD cells deficient for IFNAR, EV-A71 transcription was still inhibited by Simeprevir treatment (Fig. 3C). Moreover, when interferon α/β receptor (IFNAR) was blocked by IFNAR2-specific neutralizing antibody, the inhibitory effects of Simeprevir on ZIKV and EV-A71 were not changed in either A549 or RD cells (Fig. 3D, E, F). All these results implied that Simeprevir did not inhibit the transcription of ZIKV, EV-A71 and HSV-1 through IFN-β pathway.

Interferon-stimulated gene 15 (ISG15) belongs to ubiquitin-like (Ubl) family. It is strongly induced upon exposure to type I Interferons (IFNs), viruses, bacterial Lipopolysaccharide (LPS), and other stresses (Zhang and Zhang, 2011). Through its ISGylation process, mature ISG15 controls viral infection by regulating antiviral signaling pathways, such as RIG-I, MDA5, Mx1, PKR, filamin B, STAT1, IRF3 and

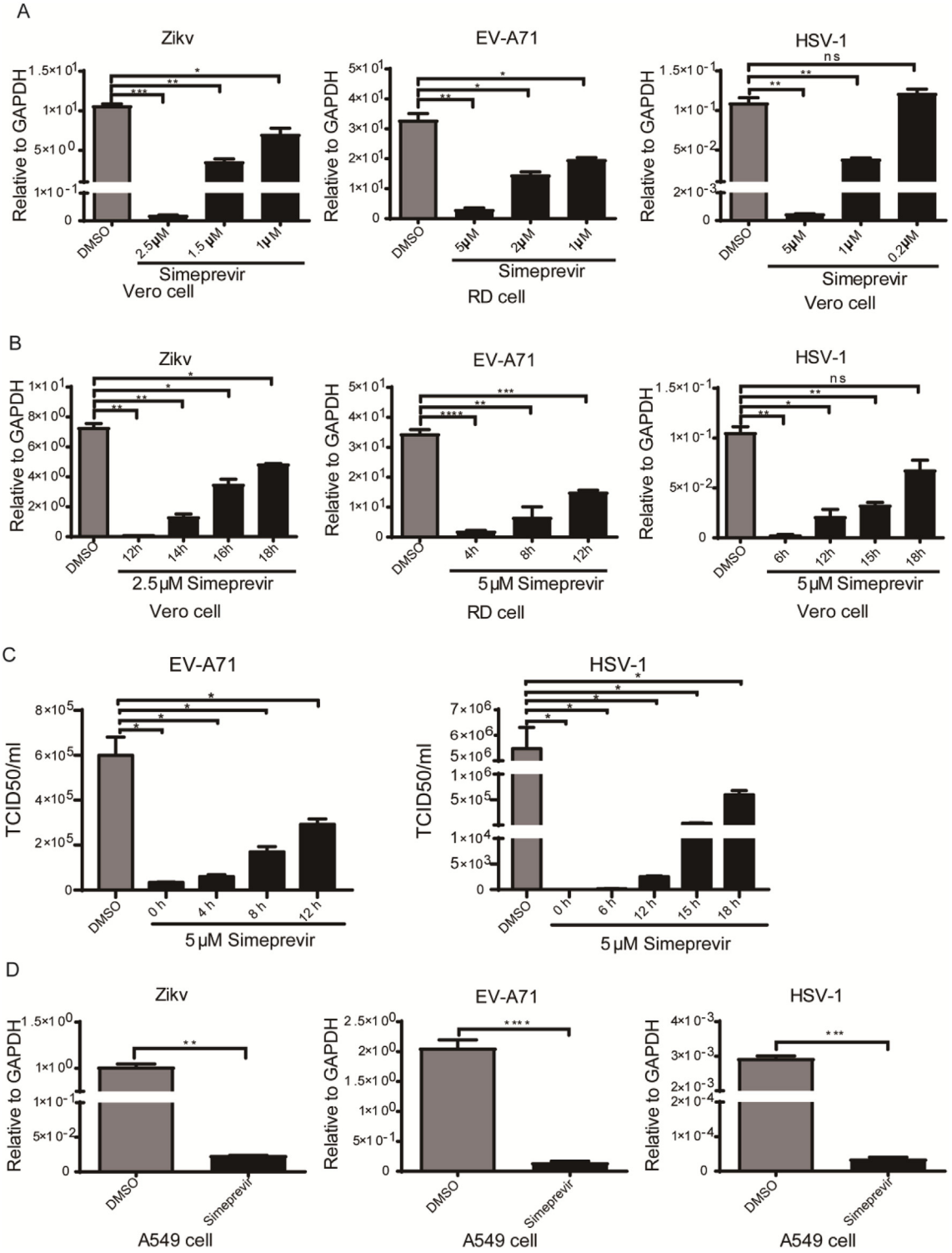
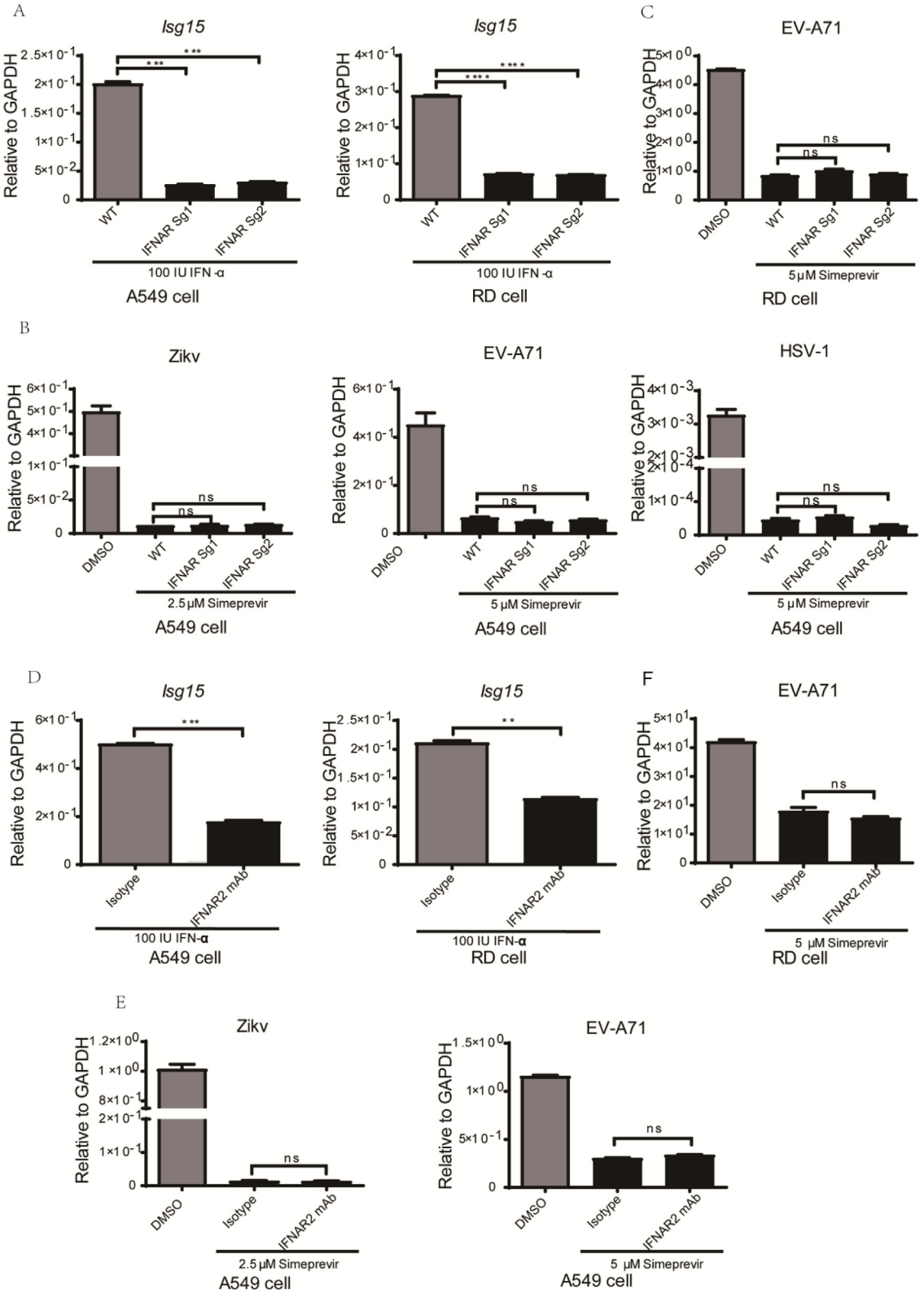


Fig. 2. Simeprevir inhibited the replication of ZIKV, EV-A71and HSV-1. (A), Different concentration of Simeprevir were add into the medium of Vero and RD cells 30 min prior to virus-infection, DMSO is the negative control. The mRNA levels of ZIKV, EV-A71 and HSV-1 were examined via real-time PCR after virus infection for 72 h (ZIKV) or 24 h (EV-A71 and HSV-1). (B), Indicated cells were treated with 2.5 μM (ZIKV) or 5 μM (EV-A71 and HSV-1) Simeprevir after initiation of virus infection for different time periods. The mRNA copies of viruses were then examined through real-time PCR after virus infection for 72 h (ZIKV) or 24 h (EV-A71 and HSV-1). (C), RD and Vero cells were infected and treated with Simeprevir as in (B), 24 h after virus infection, EV-A71 and HSV-1 virus titers were determined via TCID50 method. (D), A549 cells were treated with 2.5 μM (ZIKV) or 5 μM (EV-A71 and HSV-1) Simeprevir 30 min prior to virus infection, DMSO treatment was used as negative control; the transcription of viruses were examined through real-time PCR after viruses infection for 72 h (ZIKV) or 24 h (EV-A71 and HSV-1). The statistical differences were analyzed by t tests (and nonparametric tests). ns: not significant, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$.



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JAK1. ISG15 also conjugates some viral proteins, inhibiting viral budding and release (Harty et al., 2009). To test whether Simeprivir exerted antiviral effects directly through ISG15 bypassing IFNAR, endogenous ISG15 was silenced by ISG15-specific-ShRNA in Vero, RD and A549 cells. In such cells, IFN-α treatment could not induce ISG15 transcription anymore (Fig. 4 A). However, Simeprevir still exhibited significant antiviral effects on ZIKV, EV-A71 and HSV-1 in these ISG15-silenced cell lines (Fig. 4 B, C). All these data showed that the antiviral effects of Simeprevir were ISG15-independent.

ZIKV, EV-A71 and HSV-1 are serious pathogens that threaten human health. The last ZIKV outbreak occurred in 2015, which first appeared in Brazil, and soon spread to North America, several islands in the

Fig. 3. The inhibitory effects of Simeprevir were IFN-β-independent. IFN-α/β receptor (IFNAR) was deleted in A549 and RD cells through transducing IFNAR specific CRISPR vector (marked as IFNAR Sg1 and IFNAR Sg2), WT is the normal cells used as control. (A), To evaluate the knockout effects, the *Isg15* mRNA level in WT, IFNAR Sg1 and IFNAR Sg2 cells were detected through real-time PCR after 100 IU IFN-α treatment for 24 h. (B), After treatment of WT and IFNAR Sg1 and IFNAR Sg2 A549 cells with 2.5 μM (ZIKV) or 5 μM (EV-A71 and HSV-1) Simeprevir for 30 min, cells were infected with indicated viruses. Then the mRNA copies of viruses were examined via real-time PCR after viruses infection for 72 h (ZIKV) or 24 h (EV-A71 and HSV-1). (C), After treatment of WT and IFNAR knockout (IFNAR Sg1 and IFNAR Sg2)RD cell lines with 5 μM Simeprevir for 30 min, cells were infected with EV-A71. 24 h later, the transcription of EV-A71 was examined through real-time PCR. (D) INF-α/β receptor (IFNAR) was blocked by IFNAR2 specific antibody (marked as IFNAR2 mAb) in A549 and RD cells, IgG2a isotype (marked as isotype) was used as negative control. To evaluate the inhibitory effects, the mRNA level of *Isg15* in IFNAR2 mAb or IgG2a incubated cells were examined through real-time PCR after 100 IU IFN-α treatment for 24 h. (E), The RNA copies of ZIKV and EV-A71 were examined in A549 cells through real-time PCR after IFNAR2 blockade by antibody. (F), EV-A71 copy was measured in RD cells after IFNAR was blocked by antibody. The statistical differences were analyzed by t tests (and nonparametric tests). ns: not significant, **: $P < 0.01$, ***: $P < 0.001$ and ****: $P < 0.0001$.

Pacific Ocean, and even Southeast Asia. ZIKV infects pregnant woman, causes neurological complications, microcephaly and even death in the fetus, which has caused particular panic worldwide. EV-A71 mainly infects children under 5 years old, causes hand-foot and mouth disease, neurological paralysis and even death. HSV-1 infects a big spectrum of population, usually keeps latent but can cause a wide variety of illnesses. Up to now, there is no available vaccine and antiviral drugs to directly control these viruses. Our current study has shown that Simeprevir could effectively inhibit the transcription of ZIKV, EV-A71 and HSV-1, which may be a potential antiviral candidate for these viruses.

Simeprevir has been specifically used for hepatitis C genotype 1 and 4 by directly targeting NS3/4A protease. When it is used with medications including sofosbuvir or ribavirin and peginterferon-alfa, cure rates are in 80%–90% (Majumdar et al., 2016). It has also been reported that Simeprevir may be used in those who carry HIV/AIDS (Flanagan et al., 2014). Different from all above studies, our current work showed that Simeprevir could be used to inhibit ZIKV, EV-A71 and HSV-1, telling that Simeprevir may be extended to treat other virus infections besides HCV and HIV.

Of note, although we found that Simeprevir significantly up-regulated the transcription of IFN-β and ISG-15, the antiviral effects of Simeprevir on ZIKV, EV-A71 and HSV-1 did not rely on these molecules. Given that ZIKV, EV-A71 and HSV-1 belong to different families, Simeprevir is not likely targeting all these viruses directly. Probably other innate immune pathways have been activated by Simeprevir, which requires further work to dissect. Simeprevir has some side effects. Patients taking this medicine may feel tired, headache, rash, itchiness, and sensitivity to sunlight. These may partially attribute to its trigger of innate immune signaling. Extended research on Simeprevir may help to control those side effects in future.

1. Materials and methods

1.1. Compounds and reagents

Simeprevir was purchased from MCE (Medchem Express, CAS No.: 923604-59-5), and dissolved in DMSO (Sigma, CAS No.:67-68-5). IFN-α was obtained from R&D Systems. IFNAR2 monoclonal antibody Anti-Interferon-α/β Receptor Chain 2 Antibody, clone MMHAR-2 (MMHAR-2) and its isotype Mouse IgG2a Isotype Control from murine myeloma (M5409) were purchased from Thermo Fisher Scientific.

2. Cells lines

RD and Vero cells were cultured in DMEM (SH30243.01, HyClone) supplemented with 10% FBS (FSP500, ExCell Biology) and 1% Penicillin- Streptomycin (15140122, Thermo Fisher Scientific). A549 cells were grown in RPMI 1640 medium (SH30809, HyClone) supplemented with 10% (v/v) FBS and 1% (v/v) Penicillin-Streptomycin. A549-IFNAR$^{-/-}$ and RD-IFNAR$^{-/-}$ cell lines were generated by transfecting CRISPR/Cas9 plasmid with IFNAR specific SgRNAs (sgIFNAR1-1F/R: CACCGTAGATGACAACTTTATCCTG, AAACCAGGAT AAAG TTGTCATCTAC; sgIFNAR1-2F/R: CACCGGATCTAATGTTAAAG ACTGG, AAA CCCAGTCTTTAACATTAGATCC.). A549-Isg15-knockdown cell line was constructed by transfecting Isg15-specific-ShRNA plasmids (shRNA1: CCATGTCGG TGTCAGAGCTGA, shRNA2: GCAGA CCGTGGCCCACCTGAA.). All these cell lines were free from mycoplasma and incubated at 37 °C in 5% CO_2 incubator (Thermo Fisher Scientific).

2.1. Virus and cell infection

ZIKA virus/SZ-WIV01/2016/China was provided by the Center for Emerging Infectious Disease, Wuhan Institute of Virology, Chinese Academy of Sciences. The EV-A71 FY573 isolate (subgentype C4a, GenBank accession number: HM064456.1) have been reported before (Li et al., 2017; Wang et al., 2015). EV-A71 was propagated in RD cells, and the infectious titer was determined by $TCID_{50}$ assay. HSV-1 (KOS isolate) was kindly provided by Dr. Zhikang Qian at Institut Pasteur of Shanghai, Chinese Academy of Sciences. All virus infection assays were performed in Vero, RD and A549 cells at a multiplicity of infection (MOI) of 0.1.

Viral infection experiments followed the standard operating protocols approved by the Institutional Biosafety Committee and were performed in biosafety level 2 laboratory at Institut Pasteur of Shanghai, Chinese Academy of Sciences.

2.2. Real-time PCR

Cells were cultured and infected with virus in 12-well-plates. RNA was extracted by TriZol Reagent (15596026, Thermo), quantified by spectrophotometer (Nano-100, ALLSHENG) and reverse transcribed to cDNA using One Step PrimeScript™ RT-PCR Kit (RR064A, Takara). Real-time PCR was performed using SYBR Green Realtime PCR Master Mix (QPK-201, Toyobo) and the 7900HT Real-time PCR System (Applied Biosystems). For each sample, the normalized amount of target mRNA (N_T) was calculated from the obtained CT values of both target and GAPDH mRNA with the following equation: $N_T = 2^{CT\ of\ GAPDH - CT\ of\ target}$. The primers used are listed in Table 1.

2.3. TCID50 (50% tissue culture infectious dose) assay

After cells were infected by viruses for indicated time (24 h for EV-A71 and HSV-1), repeated freezing and thawing cycles for 3 times were applied to lyse infected RD and Vero cells to release mature EV-A71 and HSV-1 viruses. The lysed cells were diluted with 10-fold gradient in DMEM medium containing 1% FBS. RD cells (for EV-A71) and Vero cells (for HSV-1) were seeded in 96-well plates at 5000 cells/well in DMEM medium containing 10% FBS, followed with incubation at 37 °C with CO_2 overnight. Then the cell culture supernatant was discarded, and 100 μl of cell lysates containing viruses mentioned above was added to the respective wells of 96-well plates before incubation at 37 °C with CO_2 for 3–5 days. During the incubation time, cell death status was checked daily and the viral titer was calculated according to the Reed–Muench method (MUENCH, 1938).

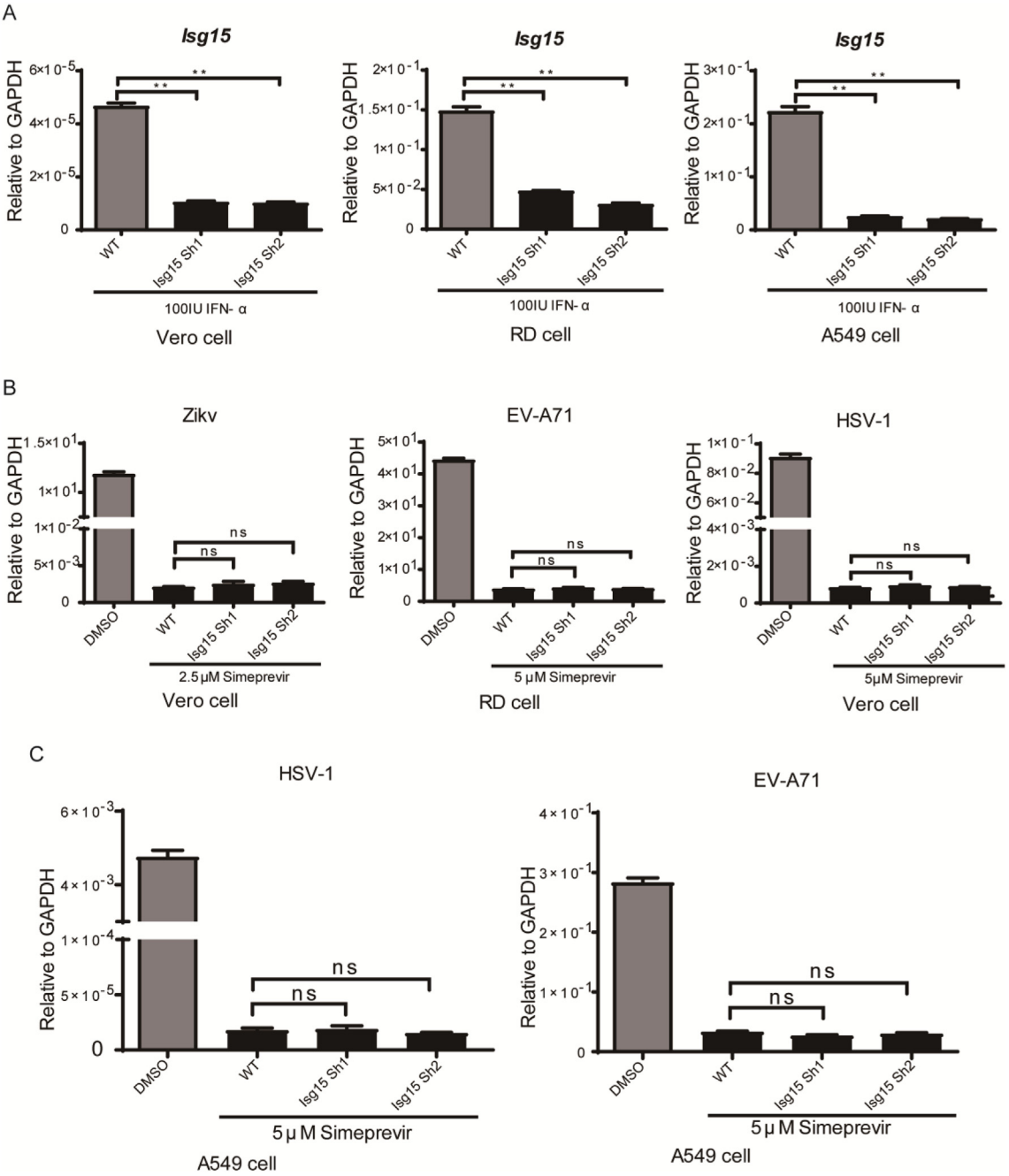

Fig. 4. The inhibitory effects of Simeprevir were ISG15-independent. *Isg15* were silenced by *Isg15* specific shRNAs (marked as Isg15 Sh1 and Isg15 Sh2), WT is the normal cells used as control. (A), To evaluate the knock-down efficiency, the *Isg1*5 mRNA level in WT, Isg15 Sh1 and Isg15 Sh2 cells were detected through real-time PCR after 100 IU IFN-α treatment for 24 h. (B), After treatment of Vero and RD WT, Isg15 Sh1 and Isg15 Sh2 cells with 2.5 μM (ZIKV) or 5 μM (EV-A71 and HSV-1) Simeprevir for 30 min, cells were infected with viruses, DMSO treated Vero and RD WT cells (marked as WT) served as controls. Real-time PCR was performed to examine the mRNA levels of ZIKV, EV-A71 and HSV-1 in Vero or RD cells after viruses infection for 72 h (ZIKV) or 24 h (EV-A71 and HSV-1). (C), The RNA copies of ZIKV and EV-A71 were examined in 5 μM Simeprevir pre-treated A549 WT, Isg15 Sh1 and Isg15 Sh2 cells after viruses infection for 24 h, DMSO treated A549 WT cells (marked as WT) served as control. The statistical differences were analyzed by t tests (and nonparametric tests). ns: not significant, **: $P < 0.01$.

Table 1
Primers used for real-time PCR.

Gene	Forward Primer	Reverse Primer
GAPDH	GGTATCGTGGAAGGACTCATGAC	ATGCCAGTGAGCTTCCCGTTCAGC
ZikV	CAACCACTGCAAGCGGAAGGGT	AAGTGATCCATGTGATCAGTTGA
EV-A71	TGAATGCGGCTAATCCCAACT	AAGAAACACGGACACCCAAAG
HSV-1	TGGGACACATGCCTTCTTGG	ACCCTTAGTCAGACTCTGTTACTTACCC
IFN-ββ	AGGACAGGATGAACTTTGAC	TGATAGACATTAGCCAGGAG
ISG15	TGGACAAATGCGACGAACCTC	TCAGCCGTACCTCGTAGGTG

2.4. Statistical analysis

GraphPad Prism 7.0 software was applied for statistical analysis. Data are presented as mean ± SEM. Comparisons between groups were performed with t tests (and nonparametric tests), and P values < 0.05 were considered statistically significant. ns: not significant, *: P < 0.05, **: P < 0.01, ***: P < 0.001 and ****: P < 0.0001.

Author contributions

G.M. conceived the project, Z.L., F.Y. and G.X. performed the experiments, Y.X., J.N., M.C., H.W., S.W. and A.L. helped with experiments, J. Z. provided critical experimental materials, G.M., Z.L., F.Y. analyzed the data and wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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