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Multiple antiviral activities of the antimalarial and anti-hepatitis C drug candidates N-89 and N-251

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ABSTRACT

The chemically synthesized endoperoxide compound N-89 and its derivative N-251 were shown to have potent antimalarial activity. We previously demonstrated that N-89 and N-251 potently inhibited the RNA replication of hepatitis C virus (HCV), which belongs to the *Flaviviridae* family. Since antimalarial and anti-HCV mechanisms have not been clarified, we were interested whether N-89 and N-251 possessed the activity against viruses other than HCV. In this study, we examined the effects of N-89 and N-251 on other flaviviruses (dengue virus and Japanese encephalitis virus) and hepatitis viruses (hepatitis B virus and hepatitis E virus). Our findings revealed that N-89 and N-251 moderately inhibited the RNA replication of Japanese encephalitis virus and hepatitis E virus, although we could not detect those anti-dengue virus activities. We also observed that N-89 and N-251 moderately inhibited the replication of hepatitis B virus at the step after viral translation. These results suggest the possibility that N-89 and N-251 act on some common host factor(s) that are necessary for viral replications, rather than the possibility that N-89 and N-251 directly act on the viral proteins except for HCV. We describe a new type of antiviral reagents, N-89 and N-251, which are applicable to multiple different viruses.

1. Introduction

The endoperoxide compound N-89 and its derivative N-251 were identified as antimalarial drug candidates by the screening of a chemically synthesized compound library [1–3]. N-89 can be cheaply synthesized from vinyl ethers and cyclododecanone by two steps [1]. N-251 can also be synthesized form bis-hydroperoxide by two steps and has higher water solubility than N-89, and both compounds are undergoing research for clinical trials [3]. On the other hand, using cell-based assay systems of hepatitis C virus (HCV), which belongs to the *Flaviviridae* family, we unexpectedly found that N-89 and N-251 potently inhibited the RNA replication of HCV [4]. Characterization of anti-HCV activities of N-89 and N-251 in that study revealed that they had potent activities at tens of nanomolar concentrations regardless of the HCV strains of genotype 1b, and suggested that anti-HCV mechanism(s) of N-89 and N-251 are different from that of interferon-α, because the anti-HCV kinetics of these compounds were faster than that of interferon-α and they exhibited synergistic effects in combination with interferon-α [4].

Recently, the sustained virologic response rate of patients with chronic hepatitis C has been improved to approximately 90% by the developed treatments with direct-acting antivirals (DAAs) [5]. We demonstrated that N-89 and N-251 had synergistic or additive anti-HCV effect when used in combination with DAAs (telaprevir, boceprevir,

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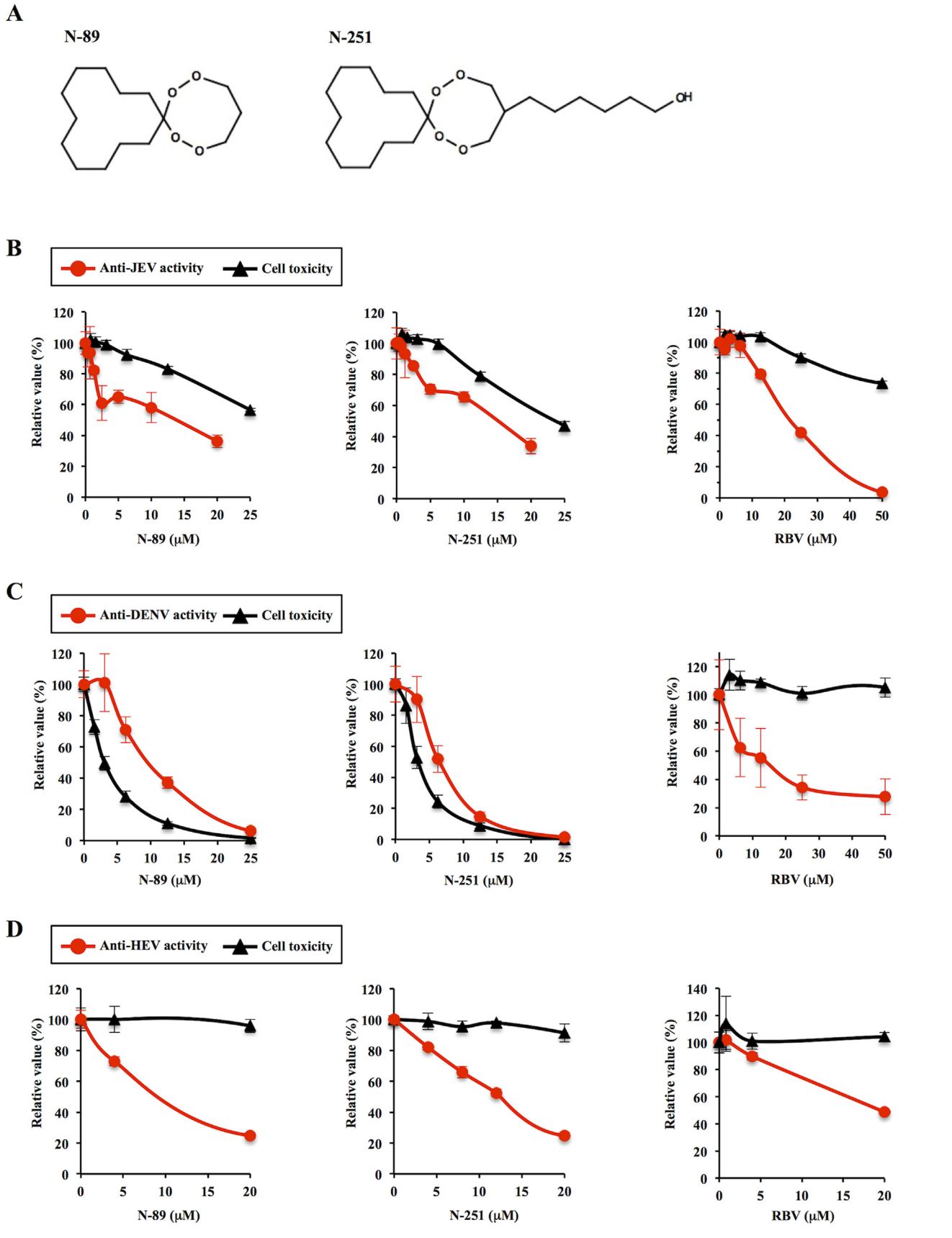

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Fig. 1. N-89 and N-251 had antiviral activities for JEV and HEV, but not DENV. **A:** Chemical formulae of N-89 and N-251. **B:** Evaluation of the anti-JEV activities of N-89, N-251, and RBV. BHKMuarPACLuc rep cells were treated with N-89, N-251, or RBV for 72 h, followed by an FL assay (*red circles*) and WST-1 cell proliferation assay (*black triangles*). The relative value (%) calculated at each point when the level in non-treated cells was used as 100% is presented. Data are mean ± SD of triplicate assays. **C:** Evaluation of the anti-DENV activities of N-89, N-251, and RBV. BHK-21 cells were treated with N-89, N-251, or RBV for 72 h, followed by a DENV/GL reporter assay (*red circles*) and WST-1 cell proliferation assay (*black triangles*). The relative value (%) calculated at each point when the level in non-treated cells was used as 100% is presented. Data are mean ± SD of triplicate assays. **D:** Evaluation of the anti-HEV activities of N-89, N-251, and RBV. PLC/PRF/5 cells treated with N-89, N-251, or RBV for 72 h, followed by an HEV/NL reporter assay and CellTiter 96® AQueous one solution cell proliferation assay. The relative value (%) calculated at each point when the level in non-treated cells was used as 100% is presented here. Data are mean ± SD of triplicate assays.

simeprevir, asunaprevir, daclatasvir, and sofosbuvir), and that N-89 and N-251 could overcome all of the DAA-resistant HCVs [6]. However, the anti-HCV mechanism(s) of N-89 and N-251 are not yet clarified. From such present conditions, we were interested whether N-89 and N-251 possessed the activity against viruses other than HCV. In this study, we here examined the effects of N-89 and N-251 on other flaviviruses and hepatitis viruses that are related to HCV. Our findings revealed multiple antiviral effects of N-89 and N-251.

2. Materials and methods

2.1. Cell cultures and reagents

BHK-21-derived BHKMuarPACLuc rep cells harboring Japanese encephalitis virus (JEV) replicon RNA were cultured with medium in the presence of puromycin (10 μg/ml) as described previously [7]. BHK-21, PLC/PRF/5, HepG2, HepG2.2.15, and HepG2/NTCPmyc cells were cultured as described previously [8–11]. N-89 and N-251 were synthesized as described previously [1–3] (Fig. 1A). Ribavirin (RBV) was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. JEV assay

The *Firefly* luciferase (FL) assay was performed to evaluate the effects of each reagent on JEV RNA replication as described previously [7]. Briefly, BHKMuarPACLuc rep cells were plated onto 24-well plates (1×10^5 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the Luciferase assay system (Promega, Madison, WI) according to the manufacturer's protocol. The 50% effective concentration (EC_{50}) of each reagent was determined from the assay results.

The WST-1 cell proliferation assay was performed to evaluate the cell toxicity of each reagent as described previously [12]. Briefly, BHKMuarPACLuc rep cells were plated onto 96-well plates (5×10^3 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. The 50% cytotoxic concentration (CC_{50}) of each reagent was determined from the assay results.

2.3. Dengue virus (DENV) assay

The DENV/*Gaussia* luciferase (GL) reporter assay was performed to evaluate the effects of each reagent on DENV RNA replication as described previously [8]. Briefly, BHK-21 cells were plated onto 24-well plates (1×10^4 cells per well) in triplicate, and then DENV replicon plasmid (DGL2) [8] was transfected to the cells. At 24 h after transfection, the cells were treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the BioLux® Gaussia luciferase assay kit (NEB, Ipswich, UK) according to the manufacturer's protocol. The EC_{50} of each reagent was determined from the assay results.

The WST-1 cell proliferation assay was performed to evaluate the cell toxicity of each reagent as described previously [12]. Briefly, BHK-21 cells were plated onto 96-well plates (5×10^2 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the WST-1 cell proliferation assay according to the manufacturer's protocol. The CC_{50} of each reagent was determined from the assay results.

2.4. Hepatitis E virus (HEV) assay

The HEV/*NanoLuc* luciferase (NL) reporter assay was performed to evaluate the effects of each reagent on HEV RNA replication as described previously [9]. Briefly, HEV replicon RNA [9] was transfected to PLC/PRF/5 cells by the electroporation method. After electroporation, the cells were plated onto 96-well plates (2.5×10^4 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the Nano-Glo® luciferase assay system (Promega) according to the manufacturer's protocol. The EC_{50} of each reagent was determined using the assay results.

The CellTiter 96® AQueous one solution cell proliferation assay (Promega) was performed to evaluate the cell toxicity of each reagent as described previously [9]. Briefly, PLC/PRF/5 cells were plated onto 96-well plates (2.5×10^4 cells per well) in triplicate and then treated with each reagent at several concentrations for 72 h. After treatment, the cells were subjected to the assay according to the manufacturer's protocol. The CC_{50} of each reagent was determined based on the assay results.

2.5. Hepatitis B virus (HBV) assay

The HBV replication assay was performed to evaluate the effects of each reagent on HBV lifecycle as described previously [11]. Briefly, HBV inoculum was prepared from the supernatant of HepG2.2.15 cells. The inoculation of HBV was performed with 1000 HBV genome equivalents per cell in culture medium containing 4% polyethylene glycol 8000 (PEG8000; Sigma-Aldrich, St. Louis, MO) and 2% dimethylsulfoxide (DMSO; Nacalai Tesque, Kyoto, Japan) for 24 h. At 24 h after the inoculation, the culture medium was replaced with fresh medium, and then cultured for 8 days for the quantitative analysis of HBV DNA. The DNAs from HBV-infected cells were prepared with an DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The quantitative polymerase chain reaction (qPCR) analysis for HBV DNA was performed using a real-time LightCycler® PCR system (Roche Diagnostics, Basel, Switzerland) as described previously [11].

The cell count was performed to evaluate the cell toxicity of each reagent as described previously [4]. Briefly, HepG2/NTCP cells were plated onto 24-well plates (1.2×10^5 cells per well) in triplicate and then treated with each reagent at several concentrations for 9 days. After treatment, the number of viable cells was counted after trypan blue dye treatment. The CC_{50} of each reagent was determined based on the assay results.

The HBV/NL reporter assay was performed to monitor the steps from the virus entry to the translation of HBV mRNAs as described previously [13–15]. Briefly, HBV/NL virus was prepared from the supernatant of HepG2 cells, into which pUC1.2HBV/NL and pUC1.2HBVΔε plasmids were cotransfected by TransIT-2020 (Mirus Bio, Madison, WI). HepG2/NTCPmyc cells were infected with the HBV/NL virus at a genome equivalent of 10–100 in the presence of 4% PEG8000 and 2% DMSO overnight. NL activity was measured using the

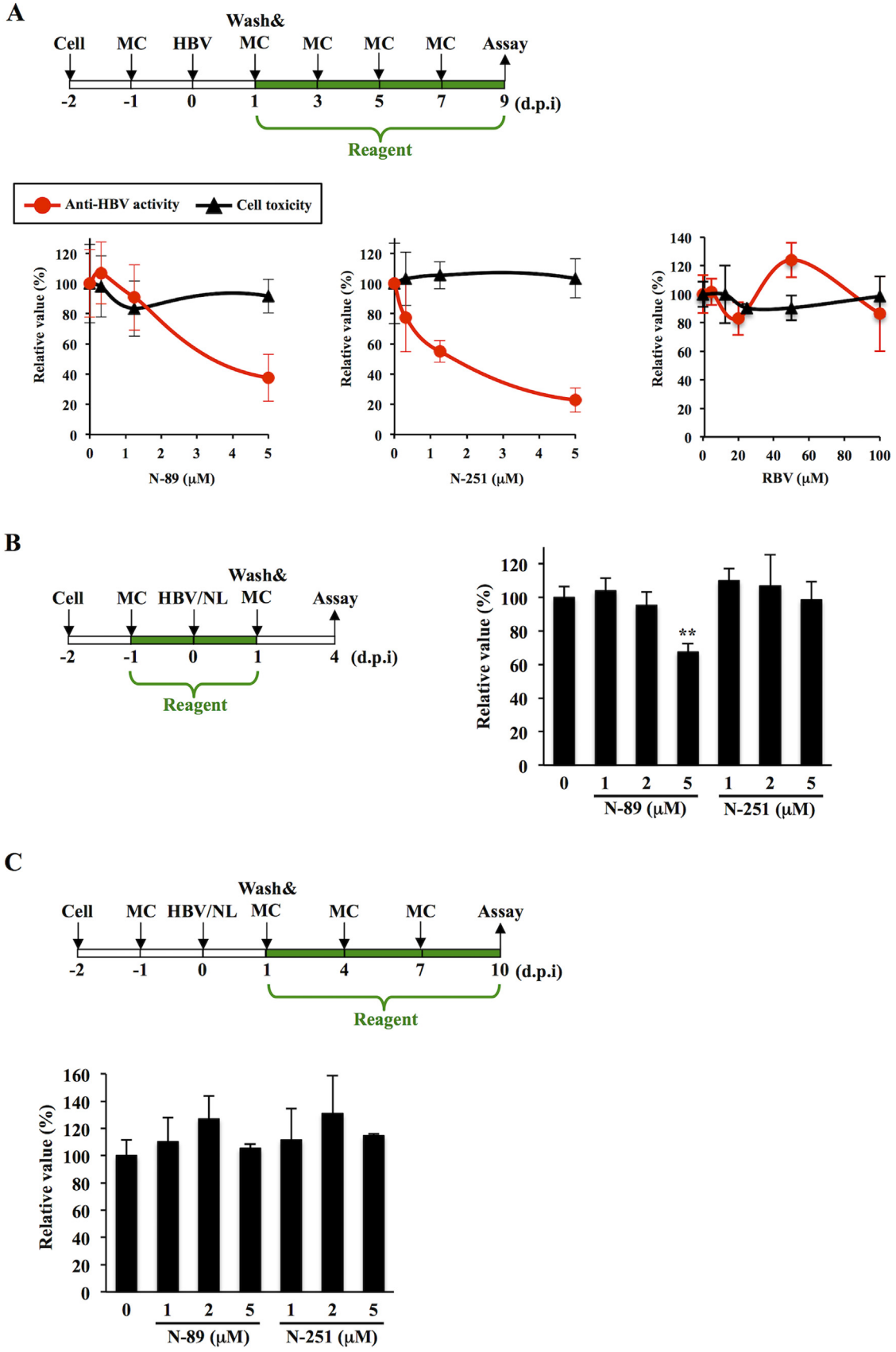

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Fig. 2. N-89 and N-251 inhibited the process after the viral translation of the HBV lifecycle. **A:** (*upper panel*) Schematic of the HBV (lifecycle) assay schedule. Wash; washing by PBS. MC; culture medium exchange. *Lower panel*: HBV-infected HepG2/NTCPmyc cells treated with N-89, N-251, or RBV followed by qPCR analysis and cell count. The relative value (%) calculated at each point when the level in non-treated cells was used as 100% is presented. Data are mean ± SD of triplicate assays. **B:** (*left panel*) Schematic of the HBV/NL reporter assay schedule (entry step). *Right panel:* HBV/NL- infected HepG2/NTCPmyc cells treated with N-89 or N-251 followed by HBV/NL reporter assay. The relative value (%) calculated at each point when the level in non-treated cells was used as 100% is presented. Data are mean ± SD of triplicate assays. ** $P < 0.01$ vs. non-treated cells. **C:** (*upper panel*) Schematic of the HBV/NL reporter assay schedule (after entry to translation steps). *Lower panel:* HBV/NL-infected HepG2/NTCPmyc cells treated with N-89 or N-251 followed by HBV/NL reporter assay.

Nano-Glo® luciferase assay system (Promega) according to the manufacturer's protocol.

2.6. Selective index (SI)

The selective index (SI) value of each reagent was determined by dividing the CC_{50} value by the EC_{50} value.

2.7. Statistical analysis

The significance of differences among groups was assessed using Student's *t*-test. Values of $P < 0.05$ were considered significant.

3. Results

3.1. N-89 and N-251 inhibited the RNA replication of JEV

Since we observed previously that N-89 and N-251 inhibited the RNA replication of HCV [4], in the present study we first examined the effects of N-89 and N-251 on JEV and DENV, which are members of the *Flaviviridae* family. In addition, we used RBV as a reference substance, because RBV is known to possess antiviral activity for a wide range of viruses including HCV, JEV, DENV, and HEV [7–9,16,17]. We evaluated the anti-JEV and anti-DENV activities of N-89, N-251, and RBV using a BHK-21-derived BHKMuarPACLuc rep assay system that can monitor the process of viral RNA replication [7] and a BHK-21-based DENV/GL reporter assay system that can monitor the process of viral RNA replication [8], respectively. The results revealed that N-89, N-251, and RBV inhibited the RNA replication of JEV (EC_{50} 14 μM; CC_{50} 28 μM; SI 2.0 for N-89, EC_{50} 15 μM; CC_{50} 24 μM; SI 1.6 for N-251, and EC_{50} 22 μM; $CC_{50} > 100$ μM; SI > 4.5 for RBV) (Fig. 1B). These results suggest that N-89 and N-251 are able to prevent the RNA replication of JEV at the same level with RBV. However, we could not detect the anti-DENV activities of N-89 and N-251 because of those relatively high cytotoxicity (EC_{50} 9.5 μM; CC_{50} 3.1 μM; SI 0.33 for N-89 and EC_{50} 6.5 μM; CC_{50} 3.4 μM; SI 0.52 for N-251) (Fig. 1C), whereas RBV inhibited the RNA replication of DENV (EC_{50} 15 μM; $CC_{50} > 100$ μM; SI > 6.7) (Fig. 1C) at the same level with previous report [8]. These results suggest that N-89 and N-251 are not suitable for the assay system of DENV.

3.2. N-89 and N-251 inhibited the RNA replication of HEV

We next examined whether N-89 and N-251 prevent the RNA replication of hepatitis viruses other than HCV. RBV was also used as a reference substance in this assay. We examined the effects of N-89, N-251, and RBV on the RNA replication of HEV, a member of the *Hepeviridae* family. We evaluated the anti-HEV activities of N-89, N-251, and RBV using a PLC/PRF/5-based HEV/NL reporter assay system that can monitor the process of viral RNA replication [9]. We found that the RNA replication of HEV was also prevented by N-89, N-251, and RBV (EC_{50} 9.2 μM; CC_{50} 59 μM; SI 6.4 for N-89, EC_{50} 13 μM; CC_{50} 56 μM; SI 4.3 for N-251, and EC_{50} 19 μM; $CC_{50} > 100$ μM; SI > 5.3 for RBV) (Fig. 1D). The values of EC_{50} were comparable to those obtained by the assay for JEV. These results showed that N-89 and N-251 possessed antiviral activities against RNA viruses belonging to a different virus family.

3.3. N-89 and N-251 inhibited the phase after viral translation in the HBV lifecycle

Since N-89 and N-251 inhibited the RNA replication of hepatitis viruses, i.e., HCV and HEV, we further examined the effects of N-89 and N-251 on HBV, a member of the *Hepadnaviridae* family. RBV was also used as a reference substance in this assay, although there is no report that RBV possesses anti-HBV activity [17]. We evaluated the anti-HBV activities of N-89, N-251, and RBV by using the HepG2/NTCP-based HBV replication assay that cover all process of virus life cycle [11] (Fig. 2A, upper panel). The results showed that HBV replication was also inhibited by N-89 and N-251 (EC_{50} 3.2 μM; CC_{50} 13 μM; SI 4.1 for N-89 and EC_{50} 1.9 μM; CC_{50} 16 μM; SI 8.4 for N-251) (Fig. 2A, lower panel), but not by RBV ($EC_{50} > 100$ μM; $CC_{50} > 100$ μM; SI < 1.0) (Fig. 2A, lower panel). The anti-HBV activity of N-251 was slightly stronger than that of N-89 (Fig. 2A, lower panel).

Since this assay system covers the entire process of HBV replication, we next examined the antiviral activities of N-89 and N-251 by using an HBV/NL reporter assay system that can monitor the steps from the virus entry to the translation of viral mRNAs. We first evaluated the antiviral activities of N-89 and N-251 in the process of virus entry (Fig. 2B, left panel). The results revealed that N-89 and N-251 did not prevent the process of virus entry, although N-89 at 5 μM slightly inhibited the virus entry (Fig. 2B, right panel).

We next evaluated the antiviral activity of N-89 and N-251 at the process of covalently closed circular DNA formation to translation of viral mRNAs (Fig. 2C, upper panel). This process was also not inhibited by N-89 or N-251 (Fig. 2C, lower panel). These results suggest that N-89 and N-251 are able to inhibit the process after the translation of virus mRNAs in the HBV lifecycle.

4. Discussion

Our present findings revealed that N-89 and N-251, which possess potent anti-HCV activity, showed moderate antiviral activities against JEV, HEV, and HBV, and that these antiviral activities took place by suppressing the replication of the virus genome as in the case of HCV. Antiviral activities of N-89, N-251, and RBV were summarized in Table 1.

Although the EC_{50} values of N-89 and N-251 to HCV were on the order of nM [4], the EC_{50} values of N-89 and N-251 to JEV, HEV, and HBV were on the order of μM (Table 1). These results suggest that the selectivity to HCV of N-89 and N-251 was higher than those to JEV, HEV, and HBV. To date, we have estimated that N-89 and N-251 might act directly on HCV proteins, which contribute to the replication of HCV RNA. As our present results demonstrated that N-89 and N-251 possess antiviral activities to three distinct viruses other than HCV, it is likely that the target(s) of N-89 and N-251 are common or analogous host factor(s) that are required for the replication of the virus genome. In addition, we estimate that N-89 and N-251 may act on both the virus and the host factors, because those anti-HCV activities are notably stronger than the antiviral activities against JEV, HEV, and HBV.

In this study, we obtained the results that N-89 and N-251 could prevent the RNA replication of JEV. However, we could not detect the anti-DENV activities of N-89 and N-251, because N-89 and N-251 were strongly cytotoxic to the cells used in DENV assay. Although JEV and DENV assays were developed using BHK-21-derived cells [7,8], the

Table 1
Antiviral activities evaluated in this study.

	N-89 (μM)			N-251 (μM)			RBV (μM)		
Virus	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI	EC_{50}	CC_{50}	SI
HCV[a,b] (HuH-7)	0.025–0.66	0.50–9.3	14–22	0.14–0.98	0.49–3.8	3.5–4.4	> 100	> 100	> 1.0
HCV[a,b] (Li23)	0.045–0.19	0.56–2.5	12–26	0.059–0.29	1.1–2.8	8.3–19	8.7–15.9	> 100	6.3– > 11
JEV	14	28	2.0	15	24	1.6	22	> 100	> 4.5
DENV	9.5	3.1	0.33	6.5	3.4	0.52	15	> 100	> 6.7
HEV	9.2	59	6.4	13	56	4.3	19	> 100	> 5.3
HBV	3.2	13	4.1	1.9	16	8.4	> 100	> 100	< 1.0

Regarding the anti-HCV activity, we divided the results of HuH-7 cell and Li23 cell-derived assays into two items, because we previously found that anti-HCV activity of RBV was greatly different between both assays [16].

[a] Results of N-89 and N-251 reported by Ueda et al. [4].

[b] Results of RBV reported by Mori et al. [16].

shape and the size of the cells used in two assays are considerably different. The difference of cell clonality may cause the cytotoxic difference observed in this study. To evaluate the effect of N-89 and N-251 to DENV fairly, new DENV assay system using the cells showing low cytotoxicity to N-89 and N-251 is necessary.

To date, since *Plasmodium falciparum* endoplasmic reticulum-resident calcium binding protein was identified as an N-251 binding protein [18], an analogous protein in humans may be involved in the antiviral activities of N-89 and N-251 observed in this study. Further analyses are needed to explore this possibility.

Malaria, HBV, HCV, and HEV are infective agents of worldwide diseases, and co-infections such as malaria/HCV and HBV/HCV have been reported [19,20]. It would thus be useful to suppress the malaria and viruses with one reagent. The usefulness of N-89 and that of N-251 in combination with HCV-specific DAAs was recently demonstrated [6], and it can thus be speculated that N-89 and N-251 would be effective in combination with other antiviral reagents. In addition, because N-89 and N-251 can be synthesized at low cost [3], these reagents will be advantageous in respect to medical expenses.

In conclusion, we have described a new type of reagents, N-89 and N-251, which possess multiple antiviral activities other than antimalarial activity.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2018.05.007.

References

[1] H.S. Kim, Y. Nagai, K. Ono, K. Begum, Y. Wataya, Y. Hamada, K. Tsuchiya, A. Masuyama, M. Nojima, K.J. McCullough, Synthesis and antimalarial activity of novel medium-sized 1,2,4,5-tetraoxacycloalkanes, J. Med. Chem. 44 (2001) 2357–2361.

[2] A. Sato, A. Hiramoto, M. Morita, M. Matsumoto, Y. Komich, Y. Nakase, N. Tanigawa, O. Hiraoka, K. Hiramoto, H. Hayatsu, K. Higaki, S. Kawai, A. Masuyama, M. Nojima, Y. Wataya, H.S. Kim, Antimalarial activity of endoperoxide compound 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol, Parasitol. Int. 60 (2011) 270–273.

[3] A. Sato, S. Kawai, A. Hiramoto, M. Morita, N. Tanigawa, Y. Nakase, Y. Komichi, M. Matsumoto, O. Hiraoka, K. Hiramoto, H. Tokuhara, A. Masuyama, M. Nojima, K. Higaki, H. Hayatsu, Y. Wataya, H.S. Kim, Antimalarial activity of 6-(1,2,6,7-tetraoxaspiro[7.11]nonadec-4-yl)hexan-1-ol (N-251) and its carboxylic acid derivatives, Parasitol. Int. 60 (2011) 488–492.

[4] Y. Ueda, M. Takeda, K. Mori, H. Dansako, T. Wakita, H.S. Kim, A. Sato, Y. Wataya, M. Ikeda, N. Kato, New preclinical antimalarial drugs potently inhibit hepatitis C virus genotype 1b RNA replication, PLoS One 8 (2013) e72519.

[5] J.M. Pawlotsky, Hepatitis C virus resistance to direct-acting antiviral drugs in interferon-free regimens, Gastroenterology 151 (2016) 70–86.

[6] Y. Ueda, H. Dansako, S. Satoh, H.S. Kim, Y. Wataya, H. Doi, M. Ikeda, N. Kato, Evaluation of preclinical antimalarial drugs, which can overcome direct-acting antivirals-resistant hepatitis C viruses, using the viral reporter assay systems, Virus Res. 235 (2017) 37–48.

[7] T. Ishikawa, M. Abe, M. Masuda, Construction of an infectious molecular clone of Japanese encephalitis virus genotype V and its derivative subgenomic replicon capable omf expressing a foreign gene, Virus Res. 195 (2015) 153–161.

[8] F. Kato, T. Kobayashi, S. Tajima, T. Takasaki, T. Miura, T. Igarashi, T. Hishiki, Development of a novel dengue-1 virus replicon system expressing secretory *Gaussia* luciferase for analysis of viral replication and discovery of antiviral drugs, Jpn. J. Infect. Dis. 67 (2014) 209–212.

[9] T. Shiota, T.C. Li, S. Yoshizaki, T. Kato, T. Wakita, K. Ishii, Establishment of hepatitis E virus infection-permissive and -non-permissive human hepatoma PLC/PRF/5 subclones, Microbiol. Immunol. 59 (2015) 89–94.

[10] M.A. Sells, M.L. Chen, G. Acs, Production of hepatitis B virus particles in HepG2 cells transfected with cloned hepatitis B virus DNA, Proc. Natl. Acad. Sci. USA 84 (1987) 1005–1009.

[11] N. Okumura, M. Ikeda, S. Satoh, H. Dansako, M. Sugiyama, M. Mizokami, N. Kato, Negative regulation of hepatitis B virus replication by forkhead box protein A in human hepatoma cells, FEBS Lett. 589 (2015) 1112–1118.

[12] Y. Ueda, K. Mori, Y. Ariumi, M. Ikeda, N. Kato, Plural assay systems derived from different cell lines and hepatitis C virus strains are required for the objective evaluation of anti-hepatitis C virus reagents, Biochem. Biophys. Res. Commun. 409 (2011) 663–668.

[13] H. Nishitsuji, S. Ujino, Y. Shimizu, K. Harada, J. Zhang, M. Sugiyama, M. Mizokami, K. Shimotohno, Novel reporter system to monitor early stages of the hepatitis B virus life cycle, Cancer Sci. 106 (2015) 1616–1624.

[14] H. Nishitsuji, H. Yamamoto, R. Shiina, K. Harada, S. Ujino, K. Shimotohno, Development of a hepatitis B virus reporter system to monitor the early stages of the replication cycle, J. Vis. Exp. 120 (2017) e54849.

[15] H. Dansako, Y. Ueda, N. Okumura, S. Satoh, M. Sugiyama, M. Mizokami, M. Ikeda, N. Kato, The cyclic GMP-AMP synthetase-STING signaling pathway is required for both the innate immune response against HBV and the suppression of HBV assembly, FEBS J. 283 (2016) 144–156.

[16] K. Mori, M. Ikeda, Y. Ariumi, H. Dansako, T. Wakita, N. Kato, Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system, Virus Res. 157 (2011) 61–70.

[17] S. Beaucourt, M. Vignuzzi, Ribavirin: a drug active against many viruses with multiple effects on virus replication and propagation. Molecular basis of ribavirin resistance, Curr. Opin. Virol. 8 (2014) 10–15.

[18] M. Morita, H. Sanai, A. Hiramoto, A. Sato, O. Hiraoka, T. Sakura, O. Kaneko, A. Masuyama, M. Nojima, Y. Wataya, H.S. Kim, *Plasmodium falciparum* endoplasmic reticulum-resident calcium binding protein is a possible target of synthetic antimalarial endoperoxides, N-89 and N-251, J. Proteome Res. 11 (2012) 5704–5711.

[19] World Health Organization, World malaria report, 2015.〈http://www.who.int/malaria/publications/world-malaria-report-2015/en/〉.

[20] World Health Organization, Global hepatitis report, 2017.〈http://www.who.int/hepatitis/publications/global-hepatitis-report2017/en/〉.