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

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A B S T R A C T

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 replication, 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 drug library [1–3]. N-89 can be cheaply synthesized from vinyl ethers and cyclododecanone by two steps [1]. N-251 can also be synthesized from 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,
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 × 10⁴ 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 50% effective concentration (EC₅₀) 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 × 10³ 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 EC₅₀ 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 × 10⁴ 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 EC₅₀ 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 × 10³ 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 CC₅₀ 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 × 10⁴ 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 NanoGlo® luciferase assay system (Promega) according to the manufacturer’s protocol. The EC₅₀ 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 × 10⁴ 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 CC₅₀ 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 NDepsey 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 × 10⁴ 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 CC₅₀ 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 pUC1.2HBVΔα 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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We next examined whether N-89 and N-251 prevent the RNA replication of HEV, a member of the Flaviviridae family. We evaluated the anti-HEV 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 HEV (EC50 14 μM; CC50 28 μM; SI 2.0 for N-89, EC50 15 μM; CC50 24 μM; SI 1.6 for N-251, and EC50 22 μM; CC50 > 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 HEV 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 (EC50 9.5 μM; CC50 3.1 μM; SI 0.33 for N-89 and EC50 6.5 μM; CC50 3.4 μM; SI 0.52 for N-251) (Fig. 1C), whereas RBV inhibited the RNA replication of DENV (EC50 15 μM; CC50 > 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 HBV

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 HBV, a member of the Flaviviridae family. 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 (EC50 14 μM; CC50 28 μM; SI 2.0 for N-89, EC50 15 μM; CC50 24 μM; SI 1.6 for N-251, and EC50 22 μM; CC50 > 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 (EC50 9.5 μM; CC50 3.1 μM; SI 0.33 for N-89 and EC50 6.5 μM; CC50 3.4 μM; SI 0.52 for N-251) (Fig. 1C), whereas RBV inhibited the RNA replication of DENV (EC50 15 μM; CC50 > 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.

3.3. 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 (EC50 3.2 μM; CC50 13 μM; SI 4.1 for N-89 and EC50 1.9 μM; CC50 16 μM; SI 8.4 for N-251) (Fig. 2A, lower panel), but not by RBV (EC50 > 100 μM; CC50 > 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/NI reporter assay system that can monitor the steps from the virus entry to the translation of viral mRNAs. We 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 EC50 values of N-89 and N-251 to HCV were on the order of nM [4], the EC50 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 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
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 a 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 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.

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