Antiviral mechanism of preclinical antimalarial compounds possessing multiple antiviral activities

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Abstract
We previously found that N-89 and its derivative, N-251, which are being developed as antimalarial compounds, showed multiple antiviral activities including hepatitis C virus (HCV). In this study, we focused on the most characterized anti-HCV activity of N-89(N-251) to clarify their antiviral mechanisms. We first prepared cells exhibiting resistance to N-89(N-251) than the parental cells by serial treatment of HCV–RNA-replicating parental cells with N-89(N-251). Then, we newly generated HCV–RNA-replicating cells with the replacement of HCV–RNAs derived from N-89(N-251)-resistant cells and parental cells. Using these cells, we examined the degree of inhibition of HCV–RNA replication by N-89(N-251) and found that the host and viral factors contributed almost equally to the resistance to N-89(N-251). To further examine the contribution of the host factors, we selected several candidate genes by cDNA microarray analysis and found that the upregulated expression of at least RAC2 and CKMT1B genes independently and differently contributed to the acquisition of an N-89(N-251)-resistant phenotype. For the viral factors, we selected several mutation candidates by the genetic comparative analysis of HCV–RNAs and showed that at least one M414I mutation in the HCV NS5B contributed to the resistance to N-89. Moreover, we demonstrated that the combination of host factors (RAC2 and/or CKMT1B) and a viral factor (M414I mutation) additively increased the resistance to N-89. In summary, we identified the host and viral factors contributing to the acquisition of N-89(N-251)-resistance in HCV–RNA replication. These findings will be useful for clarification of the antiviral mechanism of N-89(N-251).

KEYWORDS
CKMT1B, hepatitis C virus, N-89(N-251), NS5B M414I, RAC2

Abbreviations: aa, amino acid; B3GNT7, UDP-GlcNAc:betaGal beta-1,3-N-acetylgalactosaminyltransferase 7; CBB, Coomassie Brilliant Blue; CHRNA5, cholinergic receptor, nicotinic alpha 5; CKMT1B, creatine kinase, mitochondrial 1B; Con, control; CUX2, cut-like homeobox 2; DAA, direct-acting antivirals; D-PBS, Dulbecco’s phosphate-buffered saline; DPYSL3, dihydropyrimidinase-like 3; EC₅₀, 50% effective concentration; EMCV, encephalomyocarditis virus; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosome entry site; NeoR, neomycin resistance; PLA1A, phospholipase A1 member A; PPARGC1A, peroxisome proliferator-activated receptor gamma, coactivator 1 alpha; RAC2, ras-related C3 botulinum toxin substrate 2; RL, Renilla luciferase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA; SNX10, sorting nexin 10; SOCS2, suppressor of cytokine signaling 2.

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1 | INTRODUCTION

The chemically synthesized endoperoxide compound, 1,2,6,7-tetraoxaspiro [7.11] nonadecane (N-89), and its derivative, 6-(1,2,6,7-tetraoxaspiro [7.11] nonadec-4-yl) hexan-1-ol (N-251), were previously shown to have potent antimalarial activities through screening of a library of chemically synthesized compounds.1-3 N-89 has a simple structure and is synthesized from vinyl ethers and cyclodecanone in two steps.1 N-251 has higher water solubility than N-89, which can also be cheaply synthesized from bis-hydroperoxide in two steps.3 N-89 and N-251 are both undergoing clinical trials as antimalarial compounds, and are being studied from multiple perspectives to determine their effectiveness against other protozoa and their ideal dosage forms,3-5 although the antimalarial mechanism(s) of these compounds remain unclear.

Meanwhile, using cell-based reporter assay systems (ORL8 cells, OR6 cells, etc.) for hepatitis C virus (HCV)-RNA replication, we previously found that N-89 and N-251 at tens of nanomolar concentrations irrespective of the cell lines and HCV strains of genotype 1b, and found that N-89 and N-251 at the concentrations of less than 5 µM completely inhibited HCV–RNA replication.6 In addition, we have showed that the cytotoxicities of N-89 and N-251 at the concentrations of less than 5 µM was hardly observed in our cell-based reporter assay systems.6,7 Moreover, we demonstrated that these compounds exhibited synergistic effects in combination with interferon (IFN)-α, ribavirin, or various direct-acting antivirals (DAA) (daclatasvir, sofosbuvir, etc.).6,7 However, the anti-HCV mechanism(s) of N-89 and N-251 remain unresolved, although we showed that the mechanism was distinct from those of pre-existing anti-HCV drugs such as IFN-α or ribavirin.6 Recently, we found that N-89 and N-251 inhibited the RNA replication of Japanese encephalitis virus and hepatitis E virus, and inhibited the DNA replication of hepatitis B virus.8 These findings thus suggested that N-89 and N-251 exhibit broad antiviral profiles against several kinds of viruses.

To clarify the antiviral mechanism of N-89(N-251), we focused on the most characterized anti-HCV activity, because we had many experimental materials and means for this purpose, such as ORL8 or OR6 HCV–RNA-replicating cells and their cell-based reporter assay systems. In addition, as a strategy to identify the antiviral target(s) of N-89(N-251), we prepared N-89(N-251)-resistant ORL8 and OR6 cells and then, examined the cause of their resistance. Here, we report the successful identification of host and viral factors that contribute to the acquisition of an N-89(N-251)-resistant phenotype.

2 | MATERIALS AND METHODS

2.1 | Cell cultures

Two cell lines harboring HCV–RNA, human hepatoma cell line Li23-derived ORL8 cells and HuH-7-derived OR6 cells, in both of which Renilla luciferase (RL) is additionally encoded for the reporter assay (Figure 1A), were cultured with medium in the presence of G418 (0.3 mg/mL; Invitrogen, Waltham, MA) as described previously.9,10 ORL8 and OR6 cells possess the G418-resistant phenotype, because the neomycin resistance (Neo R) gene as a selective marker was produced by the efficient replication of HCV–RNA. Therefore, when HCV–RNA is excluded from the cells or when its level decreases, the cells are killed in the presence of G418.

2.2 | Reagents

N-89 and N-251 were synthesized as described previously (Figure 1B).1-3 IFN-γ was purchased from Sigma-Aldrich (St. Louis, MO).

2.3 | RL assay

The RL assay was performed as described previously.9,11,12 Briefly, the cells were plated onto 24-well plates (2 × 10⁴ cells/well) in triplicate. Each reagent at one of several concentrations was added 24 h after cell seeding. After 72 h of treatment, the cells were subjected to RL assay using an RL assay system (Promega, Madison, WI) according to the manufacturer’s protocol. From the assay results, the 50% effective concentration (EC₅₀) of each reagent was determined.

2.4 | Preparation of cured cells

To prepare cured cells, HCV–RNA-replicating cells were treated with IFN-γ as described previously.10 Briefly, the cells were treated with IFN-γ (1000 IU/mL) in the absence of G418. The treatment was repeated six times with the addition of IFN-γ at 4-day intervals. In order to determine whether or not HCV–RNA was eliminated from the cells, the cells were divided into two groups from the 4th treatment. One group of the cells was cultured in the presence of G418 (0.3 mg/mL). After the 6th treatment, we confirmed that HCV–RNA was eliminated by Coomassie Brilliant Blue (CBB) staining and Western blot analysis.
The CBB staining was performed as described previously. Briefly, the cells were washed once with 3 mL of Dulbecco’s phosphate-buffered saline (D-PBS; Sigma). Subsequently, 2 mL of CBB stain [0.6% CBB (Thermo Fisher Scientific, Waltham, MA), 50% of methanol (Nacalai Tesque, Kyoto, Japan), 10% of acetic acid (Nacalai)] was added to the dish and stirred. Finally, the cells were washed three times with 5 mL of washing solution (50% of methanol and 10% of acetic acid) and dried at room temperature.

2.5 **CBB staining**

2.6 **Western blot analysis**

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as described previously. The antibodies used in this study were HCV NS5B (a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), rat monoclonal anti-HA high affinity antibody (Roche Diagnostics, Basel, Switzerland), and β-actin (AC-15; Sigma-Aldrich), which was used as a loading control.
2.7 RNA transfection and selection of G418-resistant cells

RNA was transfected into Li23- or HuH-7-derived cured cells as described previously. Cells were selected in complete medium with G418 (0.3 mg/mL) and sodium bicarbonate solution (0.15%) for 3 weeks as described previously. The treatment was continued for 3 weeks with the addition of G418 at 4-day intervals.

2.8 Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNAs were extracted from cells using an RNeasy Mini kit (Qiagen, Hilden, Germany). The RT-qPCR analysis for HCV–RNA and host genes was performed using a real-time LightCycler®️Nano system (Roche Diagnostics) as described previously. The primer sets used in this study are shown in Table 1.

2.9 cDNA microarray analysis

Total RNAs from each cells were prepared using an RNeasy Mini kit (Qiagen). The cDNA microarray analysis was performed by the Dragon Genomics Center of Takara Bio (Otsu, Japan) using an authorized Affymetrix service provider with a GeneChip Human Genome U133 Plus 2.0 Array, as described previously.

| Gene (accession no.) | Direction | Nucleotide sequence (5’–3’) | Products (bp) |
|----------------------|-----------|-----------------------------|--------------|
| CUX2 (AB006631)      | Forward   | CCACATCTGTCTGTGAGACACAG     | 302          |
|                      | Reverse   | AGATGGCAAAGATGGTGCTGAC      |              |
| CHRNA5 (NM_000745)   | Forward   | ACATTGTTCTTCTAGACTGTCTTG    | 353          |
|                      | Reverse   | CGGATACCACAAACGTGTC        |              |
| SNX10 (NM_013322)    | Forward   | TGGGTGAGATCCTAGGATCTG       | 321          |
|                      | Reverse   | GAAGAGGTGAAGCTGCTATCAG      |              |
| CKMT1B (NM_020990)   | Forward   | CAAGAGCCAGACAATGGAATGAC     | 429          |
|                      | Reverse   | CCGGTGTATGATCCTTCATTCAC     |              |
| ENDO1 (NM_015036.20) | Forward   | TGTCCGTTCTCTGTGGATGTG       | 495          |
|                      | Reverse   | AGGTGTTGTCGTGTTTAGGATG      |              |
| B3GNT7 (NM_145236.2) | Forward   | CGGTGTCTCTCAACGAGTAGATC     | 356          |
|                      | Reverse   | TTTGACAAACACACAGGTAGAC      |              |
| DPYSL3 (NM_001387)   | Forward   | ACCT GTGAGGAATCTTCATCAGT    | 436          |
|                      | Reverse   | GCACCTACACACGTCTCACAC       |              |
| SOCS2 (NM_003877)    | Forward   | ATGTGCAAGGATAAAGGGGACAG     | 442          |
|                      | Reverse   | AAGGATCAACAGGTCTGCTGAC      |              |
| PPARGC1A (NM_013261) | Forward   | ACCAAGACAGATAGACTCTCTCTG    | 379          |
|                      | Reverse   | ATACATGCACACACAGACACTC      |              |
| OCA2 (NM_000275)     | Forward   | TGATGTTGTGCTTCCTGCAGT       | 206          |
|                      | Reverse   | CCACGTGTATGCTCTCTACATC      |              |
| P2RY2 (NM_002564)    | Forward   | CCGAGACAGATGACATGACAG       | 287          |
|                      | Reverse   | AGTTGCACTCCTGACACAGATG      |              |
| RAC2 (NM_002872.4)   | Forward   | GACTGTTCTCTCATCTGCTCTCTG    | 227          |
|                      | Reverse   | GAGTGTTGTCGCTGAGTCGATG      |              |
| PLA1A (NM_015900)    | Forward   | GGAGTTTCACTTGAAGGAACCTGAG   | 292          |
|                      | Reverse   | GTTCACTGGTCGCTGAGTAGAAGCA   |              |
| FAM43A (NM_153690.4) | Forward   | ACCTGTTGAGCCCTGCAGACTCTC    | 136          |
|                      | Reverse   | GTGTAAGTTGGGCTCCTCGTAGT     |              |
| GAPDH (NM_001256799) | Forward   | GACTCATGACCCACAGTCATG       | 334          |
|                      | Reverse   | GAGGAGACCACCTGGTGCTGAG      |              |
2.10 | RNA interference

Small interfering RNA (siRNA) targeting cut-like homeobox 2 (CUX2; M-027122-01), creatine kinase, mitochondrial 1B (CKMT1B; M-006708-01), dihydropyrimidinase-like 3 (DPYSL3; M-009821-00), suppressor of cytokine signaling 2 (SOCS2; M-017604-00), ras-related C3 botulinum toxin substrate 2 (RAC2; M-007741-01), cholinergic receptor, nicotinic alpha 5 (CHRNA5; M-006139-01), sorting nexin 10 (SNX10; M-017559-00), UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 7 (B3GNT7; M-015841-00), and phospholipase A1 member A (PLA1A; M-008411-00) were purchased from Horizon Discovery (Waterbeach, UK). Each siRNA was introduced into cells using RNAiMAX (Invitrogen). The effect of the siRNA was examined by RT-qPCR analysis. Non-targeting siRNA (D-001206-13; Horizon Discovery) was used as a negative control.

2.11 | Generation of ORL8 cells stably expressing a candidate gene

To generate ORL8 cells stably expressing a candidate gene, we introduced CKMT1B (accession no. NM_020990), DPYSL3 (accession no. NM_001387), SOCS2 (accession no. NM_003877), RAC2 (accession no. NM_002872.4), or PLA1A (accession no. NM_006139) cDNA containing a full-length ORF into the pCX4bsr/HA-tag retroviral vector as described previously. The introduced gene was confirmed by sequencing of the obtained vector. The vector was introduced into ORL8 cells using retroviral transfer by Plat-E cells. Subsequently, the cells stably expressing a candidate gene were selected using blasticidin S (10 µg/mL; Funakoshi, Tokyo). The pCX4bsr vector was also introduced into ORL8 cells as a control. The expression level of a candidate gene in the obtained cells was examined by Western blot analysis.

2.12 | Sequencing of HCV–RNA

HCV–RNA was sequenced as described previously. Briefly, to amplify the HCV–RNA, PrimeScript (Takara Bio) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT-PCR. The fragment covering the NS3 to NS5B regions (6.1 kb) was amplified and used for sequence analysis of the NS3 to NS5B regions after cloning into pBR322MC. Ten independent clones were obtained, and their nucleotide sequences were determined.

2.13 | Plasmid construction

To introduce the mutation of regions NS3 to NS5B of HCV into the plasmid pOR/3-5B QR,KE (GenBank accession no. AB191333), the Spe I to Bsi WI fragment (5711 bp, corresponding to positions 3475–9185 of the HCV genome) was removed from pOR/3-5B QR,KE. Then, the Spe I to Bsi WI fragment of the plasmid used for sequencing analysis of N-89(N-251)-resistant HCVs was introduced using a DNA Ligation Kit (Takara) according to the manufacturer’s protocol.

To introduce solely the HCV NS5B region mutation into the plasmid pOR/3-5B QR,KE, a PCR-based site-directed mutagenesis method was used as described previously. The introduced mutation was confirmed by sequencing of the obtained plasmid.

2.14 | RNA synthesis

RNA synthesis was performed as described previously. Briefly, plasmid DNA was linearized using Xba I and a MEGAscript T7 Transcription Kit (Thermo Fisher Scientific) for RNA synthesis. The synthesized RNA was dissolved in nuclease-free water.

2.15 | Transient HCV–RNA replication assay

For electroporation, 1 × 10^6 cells were suspended in 500 µL of D-PBS, and then 25 µg of Control (Con) HCV–RNA and 25 µg of the HCV–RNA possessing the various amino acid (aa) substitutions were mixed as a cell suspension in a cuvette with a gap width of 0.2 cm (Bio-Rad Laboratories, Hercules, CA). The mixture was immediately subjected to one electric pulse of 1.2 kV, 25 µF, and ∞ Ω. After electroporation, the cells were plated onto 6-well plates (1 × 10^5 cells per well) in triplicate and then, treated with each reagent for 72 h. After treatment, the cells were subjected to RT-qPCR analysis to determine the level of HCV–RNA.

2.16 | Statistical analysis

The significance of differences between groups was determined using a one-sided Student's t-test. Values of p < 0.05 were considered significant.

3 | RESULTS

3.1 | Preparation of HCV–RNA-replicating cells possessing an N-89(N-251)-resistant phenotype

Since we chose a strategy to identify the target(s) of N-89(N-251) by an analysis using N-89(N-251)-resistant
HCV–RNA-replicating cells, we first tried to prepare the cells possessing an N-89(N-251)-resistant phenotype. For this purpose, ORL8 cells and OR6 cells were continuously treated with N-251 and N-89, respectively. Although N-89(N-251)-resistant cells were not easily obtained, by means of trial and error we were able to obtain N-251-resistant ORL8 cells (named ORL8 N-251r) and N-89-resistant OR6 cells (named OR6 N-89r) by continuous treatment with N-251 (six treatments at 1 µM, followed by six treatments at 3 µM) and N-89 (six treatments at 10 µM), respectively (Figure 1C).

FIGURE 2 Both host and viral factors contributed to the acquisition of an N-89(N-251)-resistant phenotype. A, ORL8 N-251r and OR6 N-89r cells were cured by treatment with IFN-γ. The treated cells were divided into two plates with or without G418, and then cultured for 2 weeks. The obtained cells were named ORL8 N-251rc and OR6 N-89rc. The upper panels show the cells stained with CBB. The lower panels show the results of Western blot analysis of the treated and non-treated cells for HCV NS5B proteins. β-actin was used as a control for the amount of protein loaded per lane. B, G418-resistant colonies from ORL8c, ORL8 N-251rc, OR6c, or OR6 N-89rc cells transfected interchangeably with total RNAs isolated from ORL8, ORL8 N-251r, OR6, or OR6 N-89r cells. The panels show G418-resistant colonies that were stained as described in A. Each G418-resistant colonies were mixed and pooled for further analysis. The naming rules for S/R, R/S, etc. were as follows: an S or R to the left of the slash indicates whether the cell origin of introduced total RNAs was N-89(N-251)-sensitive (S) or -resistant (R), and an S or R to the right of the slash indicates whether the cells used to derive the cured cells were N-89(N-251)-sensitive (S) or -resistant (R). C, The levels of HCV–RNAs in ORL8- or OR6-derived cells. The levels of intracellular HCV–RNAs of S/S, R/S, R/R, 6S/6S, 6R/6S, 6S/6R, or 6R/6R cells were determined by RT-qPCR analysis (upper panels). Data are the mean ± SD of triplicate assays. Western blot analysis using these cells was performed as described in A (lower panels). D, Evaluation of the anti-HCV activities of N-251 in R/R, S/R, S/S, and S/S cells. S/R and S/S cells were treated with N-251 (0.125, 0.5, and 2 µM) for 72 h, R/R and R/S cells were treated with N-251 (0.25, 0.5, and 2 µM) for 72 h, and then RT-qPCR was performed as described in C (upper panel). *p < 0.05, **p < 0.01. S/S, R/S, S/R, and R/R cells were treated with N-251 (0.25, 1, and 4 µM) for 72 h, and then Western blot analysis was performed as described in A (lower panels). E, Evaluation of anti-HCV activities of N-89 in R/R, S/R, S/S, and S/S cells. R/R, S/R, and R/S cells were treated with N-89 (0.25, 0.5, and 3 µM) for 72 h, S/S cells were treated with N-89 (0.05, 0.25, 1, and 3 µM) for 72 h, and then RT-qPCR was performed as described in C (upper panel). F, Evaluation of anti-HCV activities of N-89 in 6R/6R, 6S/6R, 6S/6S, and 6R/6S cells. 6R/6R and 6S/6R cells were treated with N-89 (0.0625, 0.1, and 4 µM) for 72 h, 6R/6R and 6S/6R cells were treated with N-89 (0.25, 1, and 4 µM) for 72 h, and then RT-qPCR analysis was performed as described in C.
N-89 in ORL8 N-251r cells were 8.7-fold and 6.9-fold higher than those in parental ORL8 cells, respectively (left panels in Figure 1D), indicating that ORL8 N-251r cells possess not only an N-251-resistant phenotype, but also an N-89-resistant phenotype. We obtained similar results using OR6 N-89r cells: the EC_{50} values of N-251 and N-89 in OR6 N-89r cells were 16-fold and 12-fold higher than those in parental OR6 cells, respectively (right panels in Figure 1D). These results indicate that cells with resistance to both N-89 and N-251 can be obtained by treatment with only N-89 or only N-251, suggesting that the anti-HCV activities of N-89 and N-251 target the same or similar host or viral factor(s).

3.2 Both host and viral factors contributed to the resistance to N-89(N-251) at almost the same level

Since we obtained ORL8 N-251r and OR6 N-89r cells possessing an N-89(N-251)-resistant phenotype, we next tried to clarify whether the host or viral factor(s) contributed to the acquisition of an N-89(N-251)-resistant phenotype. For this purpose, we first prepared the exchanged cells by introducing the ORL8 N-251r or OR6 N-89r cells-derived total RNAs into ORL8c or OR6c cells, in which HCV-RNAs were eliminated by IFN-γ (Figure 2A,B). We also prepared exchanged cells by introducing the ORL8 or OR6 cells-derived total RNAs into ORL8 N-251rc or OR6 N-89rc cells, in which HCV-RNAs were eliminated by IFN-γ (Figure 2A,B). Furthermore, as a control for the comparison, we prepared the cells by re-introducing ORL8 and ORL8 N-251r cells-derived total RNAs into ORL8c and ORL8 N-251rc cells, respectively (Figure 2A,B). Similarly, we obtained the cells by re-introduced OR6 and OR6 N-89r cells-derived total RNAs into OR6c and OR6 N-89rc cells, respectively (Figure 2A,B).

In this way, we obtained each four kinds of HCV-RNA-replicating cells in each of the ORL8 or OR6 cell-based systems, and at this stage these cells were named S/S, R/S, S/R, and R/R cells for the ORL8 cell-based system and 6S/6S, 6R/6S, 6S/6R, and 6R/6R cells for the OR6 cell-based system (Figure 2B). For all of these cells, we first examined the replication levels of HCV-RNA by RT-qPCR analysis, and confirmed that the levels of HCV-RNAs in all kinds of cells were more than 10^7 copies/µg total RNA (upper panels in Figure 2C), which were equivalent to the levels in the parental ORL8 or OR6 cells.9,10 In addition, we confirmed that HCV NS5B was detected by Western blot analysis in all kinds of cells (lower panels in Figure 2C). We next evaluated the degree of N-251 resistance in R/R, S/R, R/S, and S/S cells using RT-qPCR analysis. The results revealed that the EC_{50} value of N-251 in the R/R cells [equivalent to ORL8 N-251r cells (left panels in Figure 1D)] was 5.5-fold higher than that in the S/S cells [equivalent to ORL8 cells (left panels in Figure 1D)] (upper panel in Figure 2D and Table 2). Interestingly, the EC_{50} values of N-251 in the S/R and R/S cells were 3.0-fold and 3.6-fold higher than those in S/S cells (Table 2). The differences in N-251 sensitivity observed among R/R, S/R, R/S, and S/S cells using RT-qPCR analysis. The results revealed that the EC_{50} value of N-251 in the R/R cells [equivalent to ORL8 N-251r cells (left panels in Figure 1D)] was 5.5-fold higher than that in the S/S cells [equivalent to ORL8 cells (left panels in Figure 1D)] (upper panel in Figure 2D and Table 2). Interestingly, the EC_{50} values of N-251 in the S/R and R/S cells were 3.0-fold and 3.6-fold higher than those in S/S cells (Table 2). The differences in N-251 sensitivity observed among R/R, S/R, R/S, and S/S cells were also confirmed using Western blot analysis for HCV NS5B (lower panels in Figure 2D). Similarly, we showed that the levels of N-89 resistance in the R/R, S/R, and R/S cells were 46-fold, 17-fold, and 13-fold higher than those in S/S cells (Table 2). The differences in N-251 sensitivity observed among R/R, S/R, R/S, and S/S cells were also confirmed using Western blot analysis for HCV NS5B (lower panels in Figure 2D). Similarly, we showed that the levels of N-89 resistance in the R/R, S/R, and R/S cells were 46-fold, 17-fold, and 13-fold higher than those in S/S cells (Table 2). Moreover, when we used the four kinds of cells obtained in the OR6-cell-based system, we similarly found that the levels of N-89 resistance in the 6R/6R cells were 3.0-fold, 2.6-fold, and 4.0-fold higher than that in 6S/6S cells (Figure 2F and Table 2), although the degree of N-89 resistance in the 6R/6R cells was weaker than that in the OR6 N-89r cells (right panels in Figure 1D, Figure 2F, and Table 2). Taken together, these results suggest that host factors and viral factors make nearly equivalent contributions to the acquisition of an N-89(N-251)-resistant phenotype. Since we clearly demonstrated that both host and viral factors converted ORL8 cells into an N-89(N-251)-resistant phenotype (Figure 2D,E, and

| Cells         | N-251 (µM) | Resistance (fold) | N-89 (µM) | Resistance (fold) |
|---------------|------------|-------------------|-----------|-------------------|
| S/S           | 0.20 ± 0.020 | —                 | 0.059 ± 0.011 | —                 |
| R/S           | 0.71 ± 0.041 | 3.6               | 0.79 ± 0.61 | 13                |
| S/R           | 0.59 ± 0.044 | 3.0               | 0.99 ± 0.21 | 17                |
| R/R           | 1.1 ± 0.32   | 5.5               | 2.7 ± 0.92 | 46                |
| 6S/6S         | ND          | —                 | 0.47 ± 0.18 | —                 |
| 6R/6S         | ND          | —                 | 1.9 ± 1.5  | 4.0               |
| 6S/6R         | ND          | —                 | 1.2 ± 0.68 | 2.6               |
| 6R/6R         | ND          | —                 | 1.4 ± 0.35 | 3.0               |

Abbreviation: ND, not determined.

aData are means ±95% confidence intervals from three independent experiments.

TABLE 2 Anti-HCV activities of N-89(N-251) in ORL8- or ORL8 N-251r-derived S/S, R/S, S/R, and R/R cells and in OR6 or OR6 N-89r-derived 6S/6S, 6R/6S, 6S/6R, and 6R/6R cells
Table 2), we focused on the ORL8 cell-based system in our subsequent analyses.

### 3.3 Screening of genes that may contribute to the N-89(N-251) resistance

To select the host factor(s) that contribute to the acquisition of an N-89(N-251)-resistant phenotype, we carried out a cDNA microarray analysis. To perform this analysis efficiently, we additionally prepared ORL8 cells possessing an N-89-resistant phenotype by continuous treatment with N-89 (six treatments at 1 µM, followed by six treatments at 2 µM and then, six treatments at 4 µM), and the obtained cells were named ORL8 N-89r cells (Figure 3A). We confirmed that the ORL8 N-89r cells also showed strong resistance (37-fold and 38-fold in comparison with ORL8) to both N-251 and N-89 (Figure 3B). This result indicated that the ORL8 cells obtained by the treatment with N-89 also exhibited a phenotype of resistance to both N-89 and N-251. After ORL8 N-89r cells were obtained, we performed the cDNA microarray analysis using total RNAs prepared from the parent ORL8, ORL8 N-251r, S/S, R/R, and ORL8 N-89r cells.

For the screening of upregulated genes that may contribute to N-89(N-251) resistance, we first selected genes showing an expression level of more than 100 (actual value of measurement) in N-89(N-251)-resistant cells, and then we performed three comparative analyses (ORL8 vs. ORL8 N-251r, S/S vs. ORL8 N-89r).
R/R, and ORL8 vs. ORL8 N-89r). As a result, we obtained 14 genes that exhibited more than 2-fold greater expression in N-89(N-251)-resistant cells compared with N-89(N-251)-sensitive cells (Figure 3C and Table 3).

Moreover, for the screening of downregulated genes that may contribute to N-89(N-251) resistance, we first selected genes showing an expression level of more than 100 (actual value of measurement) in N-89(N-251)-sensitive cells. Using the same method described above for the screening of upregulated genes, we obtained 13 genes whose expression levels in N-89(N-251)-resistant cells were less than 0.5-fold those in N-89(N-251)-sensitive cells (Figure 3D and Table 4).

Among these selected genes, we noticed that SOCS2, which has been reported to be involved in the replication of RNA viruses, including HCV, and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PPARGC1A), which has been shown to be a potent activator of hepatitis B virus biosynthesis, were included on the list of upregulated genes, but not on the list of downregulated genes. At this stage, since we considered that the host factor(s) might participate in the viral replication, we focused on the upregulated genes for further analysis. For each of the 14 upregulated genes, we carried out an RT-PCR analysis to check the results of the microarray analysis using the other lots of RNA specimens obtained from the five kinds of cells used in the microarray analysis. As a result, we were able to confirm the expression levels of nine genes, that is, CUX2, CHRNA5, SNX10, CKMT1B, B3GNT7, DPYSL3, SOCS2, RAC2, and PLA1A (Figure 3E), although the expression levels of other five genes were not confirmed (Figure 3E). Therefore, we used only the nine genes listed above in the subsequent analyses.

### 3.4 RAC2 or CKMT1B contributed to the N-89(N-251) resistance

To clarify that the nine genes (CUX2, CKMT1B, DPYSL3, SOCS2, RAC2, CHRNA5, SNX10, B3GNT7, and PLA1A) contribute to N-89(N-251) resistance, we first examined the effect of the knockdown of each gene by siRNA targeting on the susceptibility of the cells to N-89. S/R cells were used for this analysis, because we considered that S/R cells possessed host factor(s), but not viral factor(s), that contribute to N-89(N-251) resistance. Therefore, if an siRNA targeting such a host factor were to be introduced into S/R cells, the degree of N-89 resistance of S/R cells should be decreased. First, we confirmed by RT-qPCR analysis that each siRNA efficiently knocked down each target gene (Figure 4A). Next, we carried out RT-qPCR analysis for the HCV–RNA obtained from S/R cells that were first introduced with an siRNA and then, treated with N-89 according to the protocol shown in Figure 4B.

### Table 3 The upregulated genes that potentially contribute to the acquisition of an N-89(N-251)-resistant phenotype

| Gene                                      | Gene symbol | Sensitive cells ORL8 | ORL8 N-251r | Resistant cells ORL8 N-89r | Expression ratio |
|-------------------------------------------|-------------|----------------------|-------------|---------------------------|-----------------|
| Cut-like homeobox 2                       | CUX2        | 73  NE               | 461         | 256                       | 267             |
| Cholinergic receptor, nicotinic alpha 5   | CHRNA5      | 68  43               | 212         | 272                       | 209             |
| Sorting nexin 10                         | SNX10       | 155  67              | 449         | 356                       | 523             |
| Creatine kinase, mitochondrial 1B        | CKMT1B      | 64  63               | 213         | 278                       | 170             |
| Endonuclease domain containing 1         | ENDOD1      | 67  54               | 186         | 161                       | 262             |
| UDP-GlcNAc:betaGal beta−1,3-N-acetylgulosaminyltransferase 7 | B3GNT7 | 39  NE | 117 | 106 | 165 | 3.4 |
| Dihydropyrimidinase-like 3               | DPYSL3      | 228  NE              | 611         | 739                       | 847             |
| Suppressor of cytokine signaling 2       | SOCS2       | 293  218             | 995         | 541                       | 620             |
| Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha | PPARGC1A | 54  58 | 128 | 157 | 136 | 2.5 |
| Oculocutaneous albinism II               | OCA2        | 77  49b              | 161         | 122                       | 165             |
| Purinergic receptor P2Y, G-protein coupled, 2 | P2RY2 | 65  NE | 141 | 109 | 171 | 2.2 |
| Ras-related C3 botulinum toxin substrate 2 | RAC2 | NE NE | 153 | 162 | 235 | ND |
| Phospholipase A1 member A                | PLA1A       | NE NE                | 118         | 146                       | 209             |
| Family with sequence similarity 43, member A | FAM43A | NE NE | 111 | 132 | 110 | ND |

Abbreviations: ND, not determined; NE, judged as no expression.

Signal intensity in the human genome U133 Plus 2.0 array.

The expression ratio of each was determined by dividing the average signal intensity of resistant cells by that of sensitive cells.

Judged as marginal expression.
results revealed that knockdown of RAC2, DPYSL3, CKMTIB, PALIA, or SOCS2, but not CUX2, CHRNA5, SNX10, or B3GNT7 expression significantly decreased the degree of N-89 resistance of S/R cells (the genes are shown in their order of effectiveness) (Figure 4C). In addition, for five of these genes, that is, RAC2, DPYSL3, CKMTIB, PALIA, and SOCS2, we also observed a significant decrease of HCV–RNA level in RAC2, SOCS2, or PLA1A-knockdown cells, and the effect of RAC2 knockdown was stronger than that of SOCS2, which has been reported to be involved in the RNA replication of HCV (Figure 4D).21 No significant decrease in the HCV–RNA level in CKMT1B- or DPYSL3-knockdown cells was observed (Figure 4D). Taken together, these results suggest that RAC2, SOCS2, and PLA1A, but not CKMT1B and DPYSL3, may also participate in the RNA replication of HCV, although RAC2, SOCS2, and PLA1A have not been identified as the proviral cellular factors required for HCV replication by the analysis of genome-wide siRNA screens.24–28 In other words, these results suggest that the enhanced expressions of RAC2/SOCS2/PLA1A and CKMT1B/DPYSL3 contribute to the acquisition of an N-89(N-251)-resistant phenotype in an HCV–RNA replication-dependent and independent manner, respectively.

For the five genes remaining after the analysis using siRNA, we next examined whether overexpression of each of the five genes would convert ORL8 cells into an N-89(N-251)-resistant phenotype. First, we prepared ORL8 cells overexpressing each gene using a retrovirus expression vector, and then we confirmed by Western blot analysis that the expression level of each of the five genes in the obtained ORL8 cells was sufficient (Figure 5A). Next, we examined whether overexpression of each gene in ORL8 cells led to an N-89(N-251)-resistant phenotype in comparison with the ORL8 cells infected with the control retrovirus (named ORL8 Con). The anti-HCV activities of N-89 and N-251 in each cells were examined by RL assay. The results revealed that RAC2- or CKTM1B-overexpressing ORL8 cells were clearly converted into an N-89(N-251)-resistant phenotype (Figure 5B,C). In regard to RAC2, the EC50 values of N-251 and N-89 in ORL8 RAC2 cells became 2.6-fold and 2.1-fold higher than those in ORL8 Con cells, respectively (Figure 5B and Table 5). The results in the case of CKTM1B were similar: the EC50 values of N-251 and N-89 in ORL8 CKMT1B cells became 1.4-fold and 2.2-fold higher than those in ORL8 Con cells, respectively (Figure 5C and Table 5). In addition, we observed that PLA1A-overexpressing ORL8 cells were weakly converted into an N-89(N-251)-resistant phenotype (1.4-fold in both N-251 and N-89) (Figure 5D and Table 5). In contrast, DPYSL3-overexpressing ORL8 cells continued to display an N-89-sensitive phenotype, although they showed an N-251-resistant phenotype (1.4-fold) (Figure 5E and Table 5), and the sensitivity of N-89(N-251)

### Table 4: The downregulated genes that potentially contribute to the acquisition of an N-89(N-251)-resistant phenotype

| Gene | Gene symbol | Sensitive cells | Resistant cells |
|------|-------------|----------------|----------------|
| Cytochrome P450, family 1, subfamily A, polypeptide 1 | CYP1A1 | 7684 | 7078 |
| Anterior gradient 2, protein disulfide isomerase family member | AGR2 | 2340 | 2792 |
| Laminin, alpha 4 | LAMA4 | 949 | 1104 |
| Angiopoietin-like 3 | ANGPTL3 | 329 | 525 |
| Tyrosine aminotransferase | TAT | 1640 | 3318 |
| Neurotensin | NT5 | 4079 | 3426 |
| Solute carrier family 22, member 9 | SLC22A9 | 199 | 289 |
| Nuclear receptor subfamily 1, group H, member 4 | NR1H4 | 4117 | 1715 |
| Albumin | ALB | 24864 | 22587 |
| Alpha-2-HS-glycoprotein | AHSG | 10415 | 7208 |
| Ribosomal protein L31 | RPL31 | 163 | 113 |
| Centromere protein V pseudogene 1/2 | CENPVP1/2 | 137 | 128 |

**Abbreviations:** ND, not determined; NE, judged as no expression.

**Signal intensity in the human genome U133 Plus 2.0 array.**

**Expression ratio** determined by dividing the average signal intensity of resistant cells by that of sensitive cells.
in SOCS2-overexpressing ORL8 cells was hardly changed (Figure 5F and Table 5). Taken together, these results indicated that RAC2 or CKMT1B contributed to the acquisition of N-89(N-251) resistance, and that PLA1A weakly and DPYSL3 partially contributed to the acquisition of N-89(N-251) resistance, while SOCS2 made no contribution.

3.5 | M414I mutation of HCV NS5B contributed to the N-89 (N-251) resistance

To clarify the viral factor(s) that contribute to the N-89(N-251) resistance, we carried out a genetic analysis of HCVs derived from ORL8 N-251r cells. Since we previously...
demonstrated that N-89(N-251) inhibited the RNA replication of an HCV replicon possessing regions NS3 to NS5B. Therefore, we thought that the target(s) of N-89(N-251) would be present in the NS3 to NS5B regions. Therefore, we performed the sequencing of the NS3 to NS5B regions (approximately 6 kb) of 10 independent HCV cDNA clones (each a 6.1 kb fragment) obtained from ORL8 N-251r cells. The aa sequences deduced from the nucleotide sequences of the 10 clones analyzed were compared with the deduced consensus aa sequences of the HCV-RNAs derived from ORL8 cells (Figure 6A). For the selection of the aa substitutions that may contribute to N-89(N-251) resistance, aa substitutions derived from known adaptive mutations were first excluded from the detected aa substitutions, because adaptive mutations are known to appear naturally during the course of cell culture. Second, all of the aa substitutions that were detected in the HCV-RNAs obtained from the N-89(N-251)-sensitive Li23-derived cells during long-term cell culture were also excluded from the detected aa substitutions. However, more than 20 aa substitutions still remained as candidates even after these selections (Figure 6A).
To narrow down these aa substitutions or targeted regions for N-89(N-251) resistance, we next decided to examine the N-89 sensitivity of HCV replicon RNA possessing the sequence (NS3 to NS5B regions) of each HCV cDNA clone. To perform this analysis, the NS3 to NS5B regions of each HCV cDNA clone were replaced with the NS3 to NS5B regions of HCV replicon RNA (OR/3-5B QR,KE) (Figure 6B), and then the obtained RNA was introduced into ORL8 N-251rc cells by the electroporation method. The N-89 sensitivity of HCV replicon RNA possessing the sequences of each HCV cDNA clone was examined by transient RNA replication reporter assay, in comparison with the ORL8 N-251rc cells.

The experiments for this purpose, we first prepared ORL8 cells overexpressing both CKMT1B and/or RAC2, and then prepared the cured ORL8 CKMT1B, ORL8 RAC2, and ORL8 CKMT1/RAC2 cells by treatment with IFN-γ. The good qualities of these cured cells were confirmed by CBB staining and Western.

**FIG URE 6** M414I mutation in HCV NS5B contributed to the acquisition of an N-89(N-251)-resistant phenotype. A. Genetic analysis of HCV in ORL8 N-251rc cells. Sequence analysis of the NS3 to NS5B regions of 10 clones was performed and the resulting sequence was compared with the HCVRNA sequences obtained from parental ORL8 cells or long-term cultured Li23-derived cells. The selected and classified aa substitutions and M414I mutation. To perform the experiments for this purpose, we first prepared ORL8 cells overexpressing both CKMT1B and/or RAC2, and then prepared the cured ORL8 CKMT1B, ORL8 RAC2, and ORL8 CKMT1/RAC2 cells by treatment with IFN-γ.

**3.6 Additive effect of N-89(N-251)**

**resistance in the combination of host and viral factors**

Since we identified RAC2 and CKMT1B as parts of host factors and M414I mutation in NS5B as a part of viral factors, which are required for the acquisition of an N-89(N-251)-resistant phenotype, and each of these factors showed the effect alone, we next examined the effects in the combination of CKMT1B and/or RAC2 and M414I mutation. To perform the experiments for this purpose, we first prepared ORL8 cells overexpressing both CKMT1B and RAC2 (named ORL8 CKMT1B/RAC2 cells), and then prepared the cured ORL8 CKMT1B, ORL8 RAC2, and ORL8 CKMT1/RAC2 cells by treatment with IFN-γ. The good qualities of these cured cells were confirmed by CBB staining and Western.
blot analysis (Figure 7A). Using the obtained cells, we next evaluated the N-89 sensitivity by transient RNA replication reporter assay as described above. The ORL8 CKMT1Bc or ORL8 RAC2c cells transfected with the wt HCV replicon RNA (OR/3-5B QR,KE) significantly showed an N-89-resistant phenotype compared with ORL8c cells used as a control (Figure 7B). In addition, the ORL8 CKMT1Bc/RAC2c cells showed more resistant to N-89 than did the ORL8 CKMT1Bc or ORL8 RAC2c cells (Figure 7B), indicating that CKMT1B and RAC2 independently and additively contributed to the acquisition of an N-89-resistant phenotype. We next carried out similar experiments using HCV replicon RNA (OR/3-5B QR,KE) possessing an M414I mutation. The results revealed that the degree of N-89 resistance in the combination of the expression of CKMT1B and RAC2 and the presence of M414I mutation was enhanced more than 10-fold compared with that in parental ORL8c cells (Figure 7B). In summary, we were able to reproduce the phenomenon observed in Figures 1D, 2E, and 3B by introducing three factors (CKMT1B, RAC2, and M414I mutation) into the parental ORL8c cells, although the reproducibility was not perfect.

4 | DISCUSSION

In the present study, we prepared several kinds of HCV–RNA-replicating cells possessing an N-89(N-251)-resistant phenotype and then, using an exchange analysis of HCV–RNAs.
from N-89(N-251)-resistant cells and parental cells, we demonstrated that both host and viral factors contributed to the acquisition of an N-89(N-251)-resistant phenotype at almost the same level. Furthermore, we successfully identified at least two host factors (RAC2 and CKMT1B) and a viral factor (M414I mutation in NS5B) that contributed to the acquisition of an N-89(N-251)-resistant phenotype. Finally, we showed that the combination of both host and viral factors independently and additively enhanced the level of resistance to N-89.

One notable finding in this study is that cellular factors and viral factors changed at the same time for the acquisition of a resistant phenotype to a compound (N-89 or N-251). We observed that the host factors changed their expression levels and the viral factor mutated for the enhancement of resistance to N-89(N-251). As far as we know, this is the first report to describe a host and virus acting cooperatively to acquire resistance to an antiviral agent. In addition, from the results of the exchange analysis of HCV–RNAs from N-89(N-251)-resistant cells and parental cells, we learned that the change of expression levels of host factors was not reversible, because the cured N-89(N-251)-resistant cells (ORL8 N-251rc or OR6 N-89rc) still showed resistance to N-89(N-251), though the resistant HCVs were eliminated. However, our findings are not surprising, since it is well known that individual viruses and the cells they infect coevolve during persistent virus infection.40,41

In this study, we demonstrated that RAC2 and CKMT1B act as host factors to contribute to the resistance to N-89(N-251), although other selected host factors (PLA1A, DPYSL3, and SOCS2) also weakly or partially contribute to the resistance to N-89(N-251). RAC2 is a GTPase that contains the catalytic subunit of NADPH oxidase.42,43 In this study, we showed that enhanced expression of RAC2, which was required for HCV–RNA replication, might resist the activity of N-89(N-251) by increasing the efficiency of HCV–RNA replication. However, since enhanced expression of CUX2, which was also required for the HCV–RNA replication, did not affect the anti-HCV activity of N-89(N-251), we also considered the possibility that enhanced expression of RAC2 affects the function of other factor(s) such as RAC family members that contribute to the N-89(N-251) resistance. Moreover, CKMT1B is known to transfer high energy phosphate from mitochondria to cytosolic creatine and to be required for energy transport in mitochondria.44 In this study, we showed that CKMT1B also contributed to the resistance to N-89(N-251), although it was not necessary for the HCV–RNA replication, suggesting that it weakens the activity of N-89(N-251) by catching N-89(N-251). Since there has been a report on the connection between RAC2 and CKMT1B, RAC2 and CKMT1B independently and differently act for the acquisition of an N-89(N-251)-resistant phenotype. In fact, we observed that RAC2 and CKMT1B additively enhanced the resistance to N-89(N-251) (Figure 7B). Moreover, since RAC2 has been reported to bind directly to several compounds with low molecular weights,45,46 we were not able to exclude the possibility that RAC2 directly binds to N-89(N-251) and interferes with its anti-HCV activity. Further analysis on direct binding of N-89(N-251) to RAC2 or CKMT1B will be needed. Moreover, there may be the possibility that HA-tagged gene(s) lose its function in cells, and, as a result, such gene(s) does not show the significant increase of EC50 value compared to the control cells. In addition, although we examined only the upregulated genes (Table 3) at this time, it is likely that other genes contributing to N-89(N-251) resistance are obtained from the downregulated genes (Table 4). Further studies will also be needed to clear this point and to elucidate the action mechanisms of RAC2 and CKMT1B.

As a viral factor involved in N-89(N-251) resistance, M414I mutation in NS5B was identified by an analysis using a point mutagenesis method (Figure 6E). Position 414 is located in a thumb domain of NS5B (Figure 6F) and is predicted to act as a primer buttress helix.39 In fact, M414 T, but not M414I, is known to be a resistance-associated substitution to the non-nucleoside NS5B polymerase inhibitor, dasabuvir.47 Therefore, N-89(N-251) may also exert its anti-HCV activity as a DAA to NS5B in the manner of dasabuvir. Since we previously showed that N-89(N-251) exhibited synergistic effects in combination with existing DAAs and could overcome DAA-resistant HCVs,7 N-89(N-251) may be a new type of DAA against HCV NS5B polymerase. In addition, we detected that I in position 414 is actually present in patients with untreated chronic hepatitis C of genotype 1b (GenBank accession nos. KT873022 and EU155373, etc.). This information may be useful for pretreatment diagnosis. Moreover, we detected many aa substitutions as candidates in the C-terminal region of NS5A by the comparative sequence analysis of HCVs derived from N-89(N-251)-resistant ORL8 N-251r cells (Figure 6A). Although this region of NS5A is known to be dispensable for the replication of HCV–RNA37 and shows wide variation among HCV clones,36 we are not able to exclude the possibility that this region contributes to the acquisition of an N-89(N-251)-resistant phenotype. Future analysis of this region will be needed.

Although we were not able to prove the direct binding of N-89 to NS5B or CKMT1B, we have provided schematic diagrams (Figure 7C) to illustrate one of the possible mechanisms of the conversion from N-89(N-251)-sensitive cells into N-89(N-251)-resistant cells. In this model, a mutation in NS5B and enhanced expression of RAC2 and CKMT1B independently and differently act for the acquisition of an N-89(N-251)-resistant phenotype. We previously reported that N-89(N-251) inhibited the RNA replication of Japanese encephalitis virus and hepatitis E virus, and the DNA replication of hepatitis B virus,8 and this model might be applied.
to these viruses. In addition, it is likely that several factors other than those identified in this study are also targets of N-89(N-251). To evaluate these possibilities, further analysis using these viruses will be needed.

In conclusion, we identified both host and viral factors leading to the conversion from an N-89(N-251)-sensitive phenotype to the resistant phenotype using HCV–RNA-replicating cells. Our results should assist in further clarification of the antiviral mechanisms of N-89(N-251).

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CONFLICT OF INTEREST
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS
W. Gu, Y. Ueda, and N. Kato designed research; W. Gu and Y. Ueda performed research; W. Gu, Y. Ueda, H. Dansako, S. Satoh, and N. Kato contributed new reagents or analytic tools; W. Gu, Y. Ueda, H. Dansako, S. Satoh, and N. Kato analyzed data; and W. Gu, Y. Ueda, and N. Kato wrote the paper.

DATA AVAILABILITY STATEMENT
All data generated or analyzed during this study are included in the published article.

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