Received: 27 October 2020 | Revised: 18 January 2021 | Accepted: 9 February 2021

DOI: 10.1096/fba.2020-00107

FASEB BioAdvances WILEY

RESEARCH ARTICLE

Antiviral mechanism of preclinical antimalarial compounds possessing multiple antiviral activities

Weilin Gu | **Youki Ueda** | **Hiromichi Dansako** | **Shinya Satoh** | **Nobuyuki Kato**

Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan

Correspondence
Dr. Nobuyuki Kato, Department of Tumor Virology, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, 2-5-1 Shikata-cho, Okayama 700-8558, Japan.
Email: nkato@md.okayama-u.ac.jp

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-acetylglucosaminyltransferase 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; EC_{50}, 50% effective concentration; EMCV, encephalomyocarditis virus; *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosome entry site; Neo^R, 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.

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 cyclododecanone 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 (2×10^4 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 (EC_{50}) 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 4^{th} treatment. One group of the cells was cultured in the presence of G418 (0.3 mg/mL). After the 6^{th} treatment, we confirmed that HCV–RNA was eliminated by Coomassie Brilliant Blue (CBB) staining and Western blot analysis.

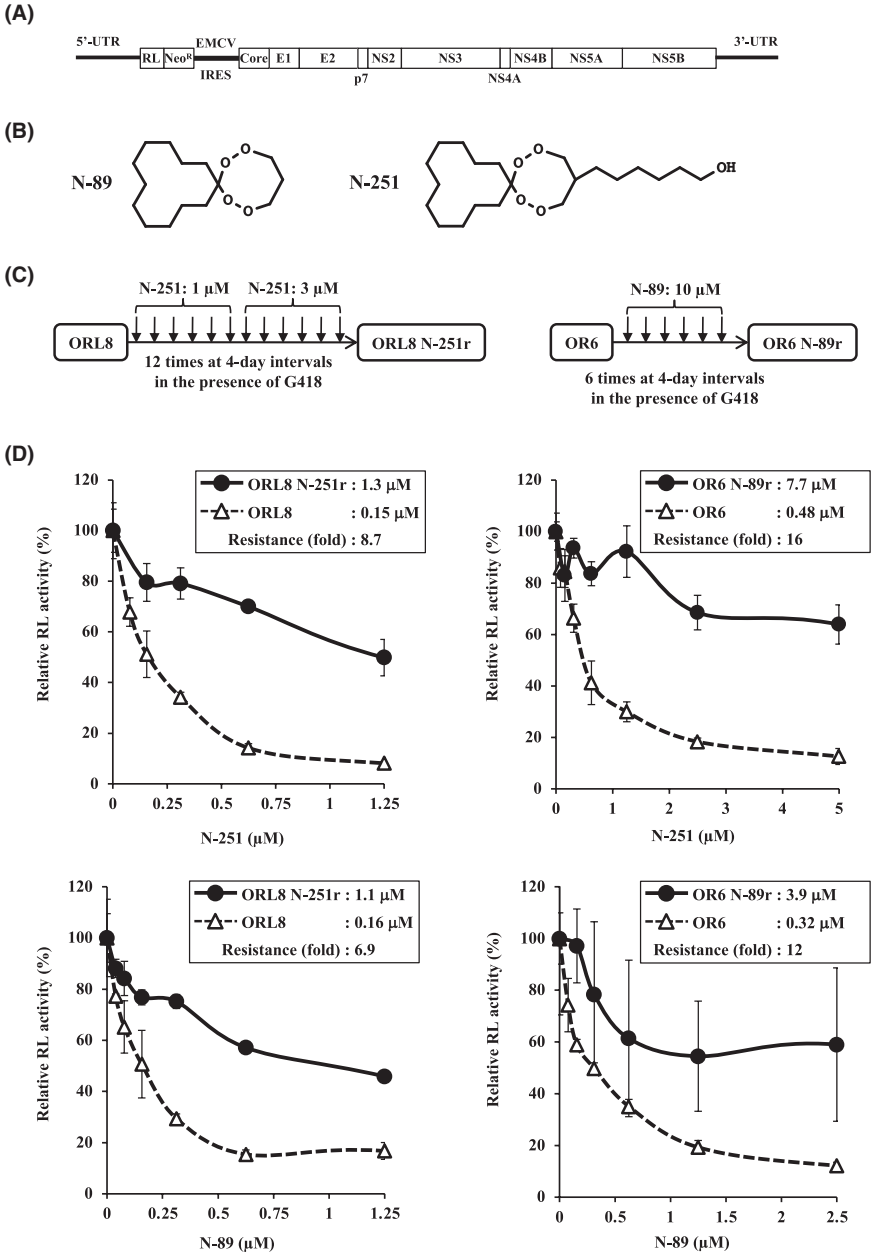


FIGURE 1 Characterization of ORL8- or OR6-derived cells possessing an N-89(N-251)-resistant phenotype. A, Structure of replicable HCV–RNA used for the reporter assay. The RL, Neo^R, and encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) are additionally introduced to the full-length HCV–RNA. B, Structures of N-89 and N-251. C, Outline for the preparation of HCV–RNA-replicating cells possessing an N-251- or N-89-resistant phenotype (ORL8 N-251r or OR6 N-89r cells). D, Evaluation of anti-HCV activities of N-251 and N-89 in ORL8 N-251r and OR6 N-89r cells. ORL8, OR6 cells (open triangles) and ORL8 N-251r, OR6 N-89r cells (black circles) were treated with N-251 (upper panels) or N-89 (lower panels) for 72 h, followed by RL assay. The relative value (%) calculated at each point when the level in non-treated cells was used as 100% is presented. Data are the mean ±SD of triplicate assays. EC_{50} values of N-251 and N-89 in ORL8 N-251r and OR6 N-89 cells were determined by other assays using N-251 and N-89 at concentrations higher than those used here

2.5 | CBB staining

The CBB staining was performed as described previously.[9] 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.

2.6 | Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and immunoblotting analysis were performed as described previously.[9] 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.[13] Cells were selected in complete medium with G418 (0.3 mg/mL) and sodium bicarbonate solution (0.15%) for 3 weeks as described previously.[14] 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.[15] 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.[10,16,17]

TABLE 1 The primers used for RT-qPCR analysis

Gene (accession no.)	Direction	Nucleotide sequence (5'−3')	Products (bp)
CUX2 (AB006631)	Forward	CCACATCTGTCTGTGAGACACAG	302
	Reverse	AGATGGACAAGATGGATGTCTGAC	
CHRNA5 (NM_000745)	Forward	ACGTGTTCCTTCAGACTCTGTCTG	353
	Reverse	CGGAT ACCAGCAACAGCTGTC	
SNX10 (NM_013322)	Forward	TGGGTTCGAGATCCTAGGATTCAG	321
	Reverse	GAAGAGGTGAAGGCTGCTATCTG	
CKMT1B (NM_020990)	Forward	CAAGAGCGACACAATGGATATGAC	429
	Reverse	CCGTGTATGATCCTCCTCATTCAC	
ENDOD1 (NM_015036.20)	Forward	TGTCCGTGTCCTTGTGGATGTG	495
	Reverse	AGGTGGTCGTCTGTGTTAGAGTG	
B3GNT7 (NM_145236.2)	Forward	CGGTGTTCCAACGCAGTCTCAC	356
	Reverse	CTTGACAACCACCAGCAGGTAGAC	
DPYSL3 (NM_001387)	Forward	ACCTGTGAGGAATCTTCATCAGTC	436
	Reverse	GCACTACACACGCTCTCAACAC	
SOCS2 (NM_003877)	Forward	ATGTGCAAGGATAAGCGGACAG	442
	Reverse	AAGGATCAACAGGTCTTGGTCAG	
PPARGC1A (NM_013261)	Forward	ACCAAGAGCAAGTATGACTCTCTG	379
	Reverse	ATACATGCACACACGCACACTC	
OCA2 (NM_000275)	Forward	TGATGGTTGTGTCCTGCACTG	206
	Reverse	CCACTGTGTCTCTGTAGCATCTC	
P2RY2 (NM_002564)	Forward	CCGACAGAACTGACATGCAGAG	287
	Reverse	AGTTGACTCTGGACCACAGAGTG	
RAC2 (NM_002872.4)	Forward	GACGTCTTCCTCATCTGCTTCTC	227
	Reverse	GAGTCAATCTCCTTGGCCAGTG	
PLA1A (NM_015900)	Forward	GGAGTTTCACTTGAAGGAACTGAG	292
	Reverse	GTTCACTGGTTCAGGTAAGCAGAC	
FAM43A (NM_153690.4)	Forward	ACGCTGTGAGCCTGCACTACTC	136
	Reverse	GTGTAAGTTGGGTCCTCGCTAGTG	
GAPDH (NM_001256799)	Forward	GACTCATGACCACAGTCCATGC	334
	Reverse	GAGGAGACCACCTGGTGCTCAG	

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 form 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_015900) cDNA containing a full-length ORF into the pCX4bsr/HA-tag retroviral vector as described previously.[18] The introduced gene was confirmed by sequencing of the obtained vector. The vector was introduced into the 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.[9,10,14] 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.[14] 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)[9] 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.[7,19] The introduced mutation was confirmed by sequencing of the obtained plasmid.

2.14 | RNA synthesis

RNA synthesis was performed as described previously.[7] 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

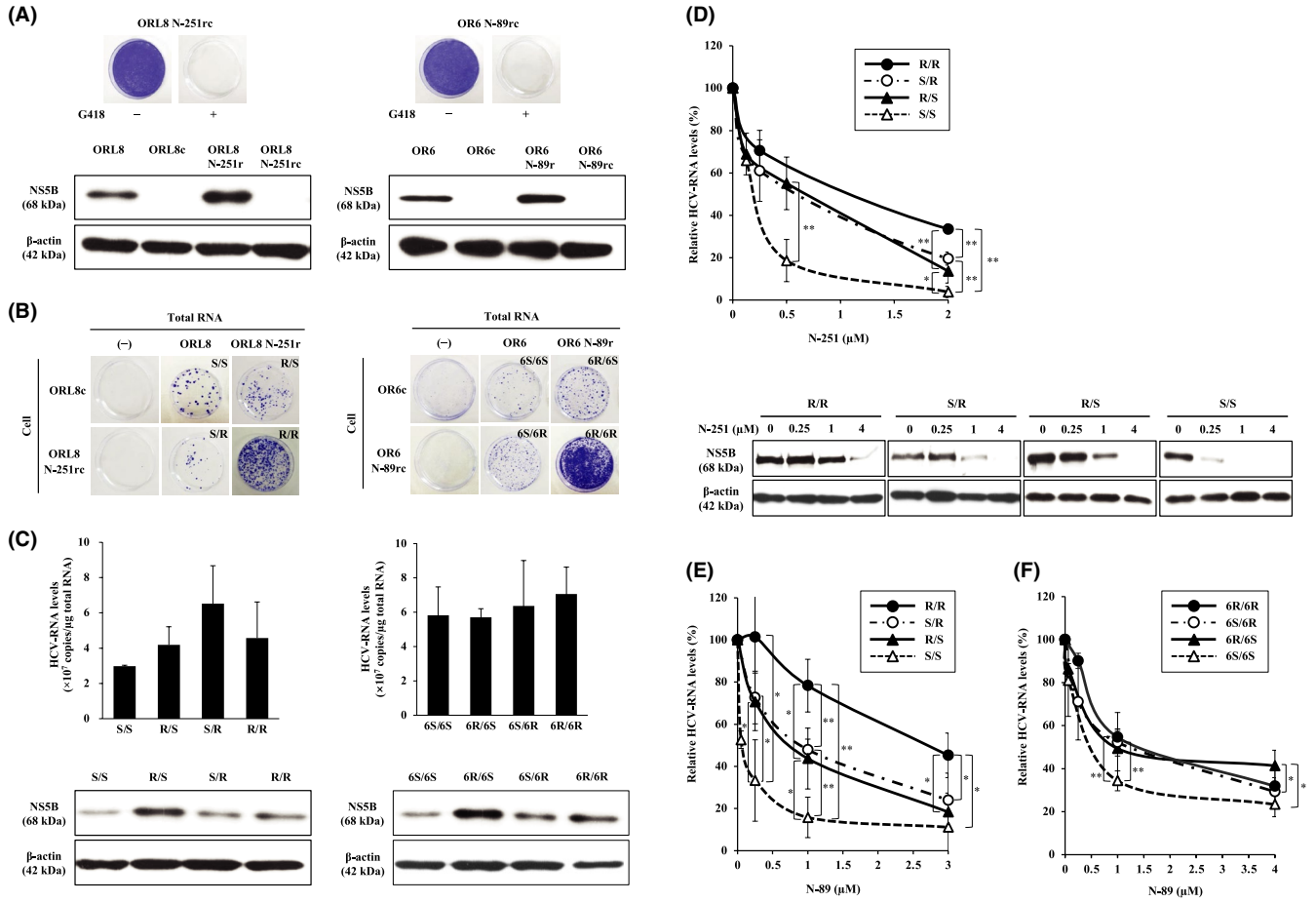


FIGURE 2 Both host and viral factors contributed to the acquisition of an N-89(N-251)-resistant phenotype. A, ORL8 N-251r and OR6 N-89r cells were cured by treatment with IFN-γ. The treated cells were divided into two plates with or without G418, and then cultured for 2 weeks. The obtained cells were 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, S/R, 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, R/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 analysis 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, R/S, and S/S cells. R/R, S/R, and R/S cells were treated with N-89 (0.25, 1, 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 analysis was performed as described in C. F, Evaluation of anti-HCV activities of N-89 in 6R/6R. 6S/6R, 6R/6S, and 6S/6S cells. 6R/6S and 6S/6S 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

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). Next, using an RL assay we evaluated the degree of resistance to N-89 and N-251 of ORL8 N-251r and OR6 N-89r cells by comparison with parental ORL8 and OR6 cells. The results revealed that the EC_{50} values of N-251 and

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

Cells	N−251 (μM)		N−89 (μM)	
	EC_{50} [a]	Resistance (fold)	EC_{50} [a]	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.

[a]Data are means ±95% confidence intervals from three independent experiments.

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[10] or OR6c[9] 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 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 that in S/S cells (Figure 2E and 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, 6S/6R, and 6R/6S 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

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.

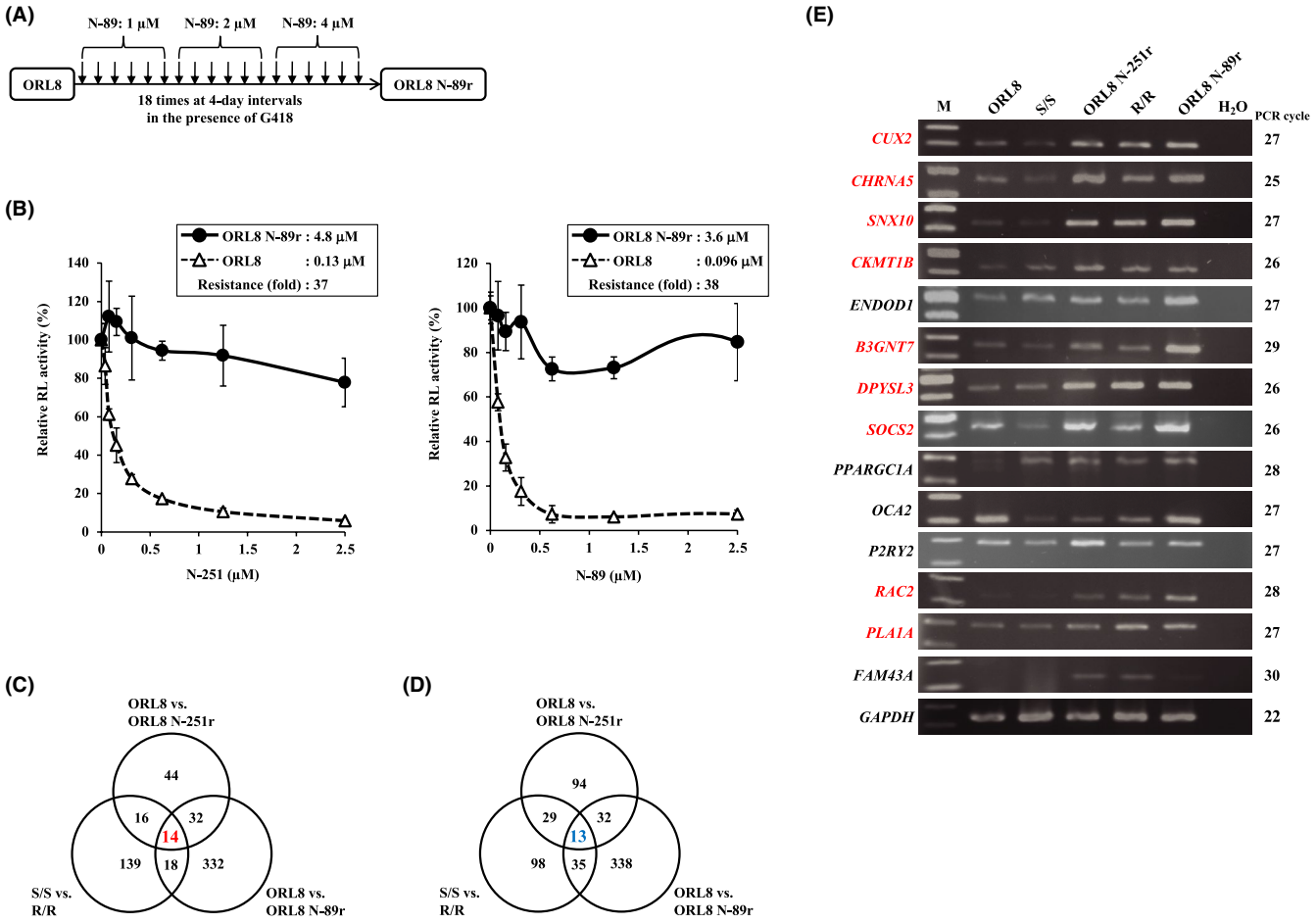


FIGURE 3 Screening of genes that may contribute to the acquisition of an N-89(N-251)-resistant phenotype. A, Outline for the preparation of HCV–RNA-replicating cells (ORL8 N-89r) possessing an N-89-resistant phenotype. B, Evaluation of anti-HCV activities of N-251 (left panel) and N-89 (right panel) in ORL8 N-89r cells. ORL8 (open triangles) and ORL8 N-89r (black circles) cells were treated with N-251 or N-89 for 72 h, and then an RL assay was performed as described in Figure 1D. C, Genes whose expression levels in ORL8-derived N-89(N-251)-resistant cells were more than 2-fold greater than their expression levels in N-89(N-251)-sensitive cells were selected. Fourteen genes, all of which were upregulated commonly in all three comparisons, were obtained. D, Genes whose expression levels were downregulated at an average ratio of less than 0.5 in the case of each ORL8-derived N-89(N-251)-resistant cells versus N-89(N-251)-sensitive cells were selected. Thirteen genes, all of which were downregulated commonly in all three comparisons, were obtained. E, Expression profiles of 14 genes that were upregulated in each of the cell lines used for microarray analyses were examined by RT-PCR analysis. PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. M, molecular weight marker

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,[20-22] and peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (*PPARGC1A*), which has been shown to be a potent activator of hepatitis B virus biosynthesis,[23] 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. The

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

Gene	Gene symbol	Sensitive cells		Resistant cells			Expression ratio[a] (fold)
		ORL8	S/S	ORL8 N−251r	R/R	ORL8 N−89r	
Cut-like homeobox 2	*CUX2*	73	NE	461	256	267	4.5
Cholinergic receptor, nicotinic alpha 5	*CHRNA5*	68	43	212	272	209	4.2
Sorting nexin 10	*SNX10*	155	67	449	356	523	4.0
Creatine kinase, mitochondrial 1B	*CKMT1B*	64	63	213	278	170	3.5
Endonuclease domain containing 1	*ENDOD1*	67	54	186	161	262	3.4
UDP-GlcNAc:betaGal beta−1,3-N-acetylglucosaminyltransferase 7	*B3GNT7*	39	NE	117	106	165	3.4
Dihydropyrimidinase-like 3	*DPYSL3*	228	NE	611	739	847	3.2
Suppressor of cytokine signaling 2	*SOCS2*	293	218	995	541	620	2.8
Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	*PPARGC1A*	54	58	128	157	136	2.5
Oculocutaneous albinism II	*OCA2*	77	49[b]	161	122	165	2.4
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	ND
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.

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

[b]Judged as marginal expression.

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			Expression ratio[a] (fold)
		ORL8	S/S	ORL8 N−251r	R/R	ORL8 N−89r	
Cytochrome P450, family 1, subfamily A, polypeptide 1	*CYP1A1*	7684	7078	2675	2270	422	0.24
Anterior gradient 2, protein disulfide isomerase family member	*AGR2*	2340	2792	1007	645	386	0.26
Laminin, alpha 4	*LAMA4*	949	1104	232	240	445	0.30
Angiopoietin-like 3	*ANGPTL3*	329	525	140	121	NE	0.31
Tyrosine aminotransferase	*TAT*	1640	3318	716	1570	149	0.33
Neurotensin	*NTS*	4079	3426	1854	1286	760	0.35
Solute carrier family 22, member 9	*SLC22A9*	199	289	NE	90	NE	0.37
Nuclear receptor subfamily 1, group H, member 4	*NR1H4*	4117	1715	1452	739	1363	0.41
Albumin	*ALB*	24864	22587	9616	10185	10140	0.42
Alpha−2-HS-glycoprotein	*AHSG*	10415	7208	4702	2938	4943	0.48
BPI fold containing family A, member 4, pseudogene	*BPIFA4P*	220	359	NE	NE	NE	ND
Ribosomal protein L31	*RPL31*	163	113	NE	NE	NE	ND
Centromere protein V pseudogene 1/2	*CENPVP1/2*	137	128	NE	NE	NE	ND

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

Signal intensity in the human genome U133 Plus 2.0 array.

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

results revealed that knockdown of *RAC2*, *DPYSL3*, *CKMT1B*, *PAL1A*, 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*, *CKMT1B*, *PAL1A*, 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 EC_{50} 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 EC_{50} 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)

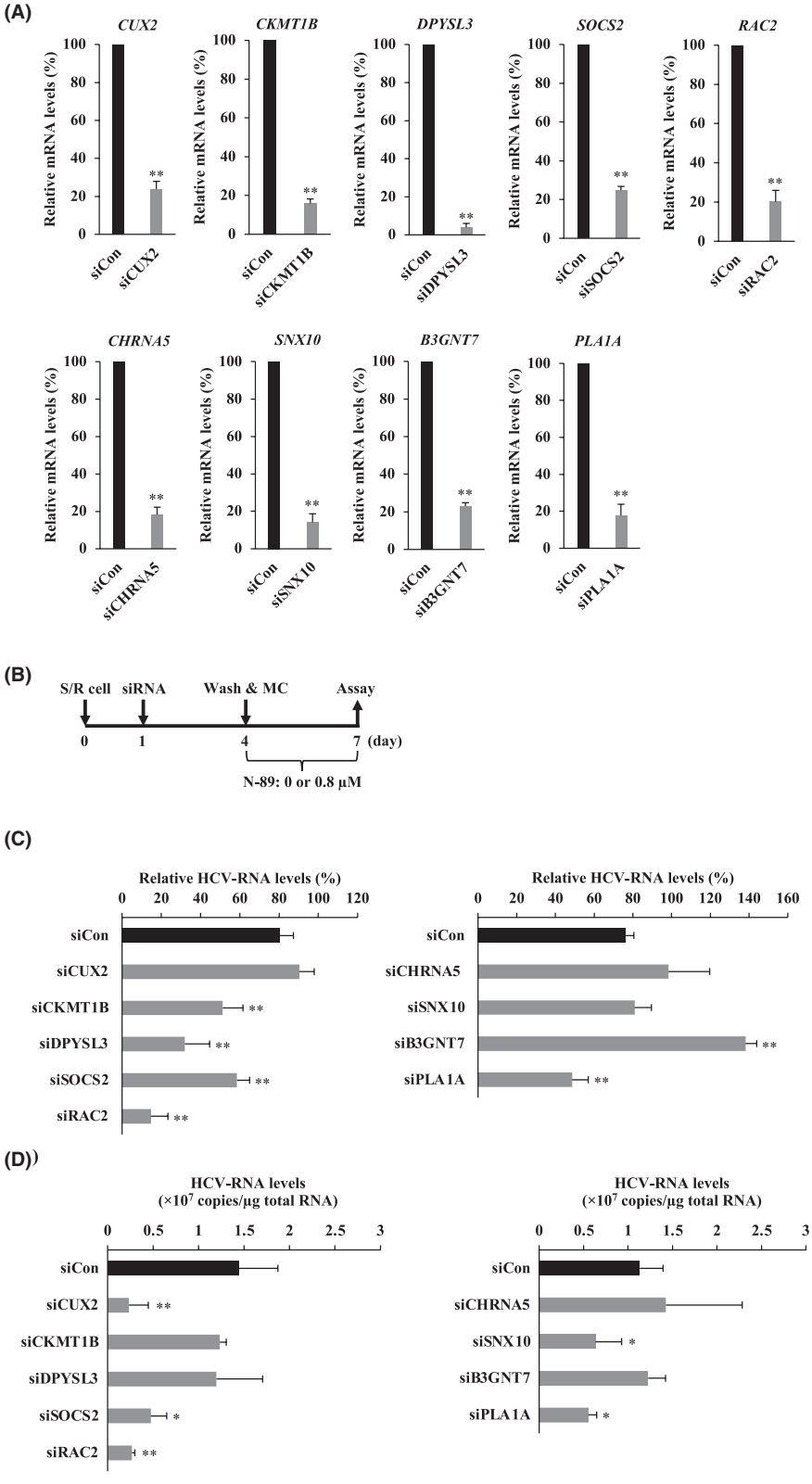


FIGURE 4 The suppression of each of 5 genes in S/R cells canceled the N-89-resistant phenotype. A, Intracellular mRNA level of each gene in siRNA-transfected cells. S/R cells transfected with 0.2 nM siCUX2, siCKMT1B, siDPYSL3, siSOCS2, or siRAC2 or 0.5 nM siCHRNA5, siSNX10, siB3GNT7, siPLA1A, or siCon were cultured for 72 h, and then the intracellular mRNA level of each gene was determined by RT-qPCR analysis. The relative value (%) calculated at each time point when the level in siCon-transfected cells was taken as 100% is presented. Data are the mean ± SD of triplicate assays. $^{**}p < 0.01$. B, Outline of the evaluation of anti-HCV activity in each gene knockdown of S/R cells. C, Evaluation of anti-HCV activity in each gene knockdown of S/R cells. RT-qPCR analysis was performed as described in Figure 2C. The relative value (%) calculated for each time point when the level in non-treated cells was taken as 100% in presented. Data are the mean ±SD of triplicate assays. $^{*}p < 0.05$, $^{**}p < 0.01$ versus siCon-transfected cells. D, The levels of HCV–RNAs in the knockdown cells for each gene were examined by RT-qPCR analysis

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

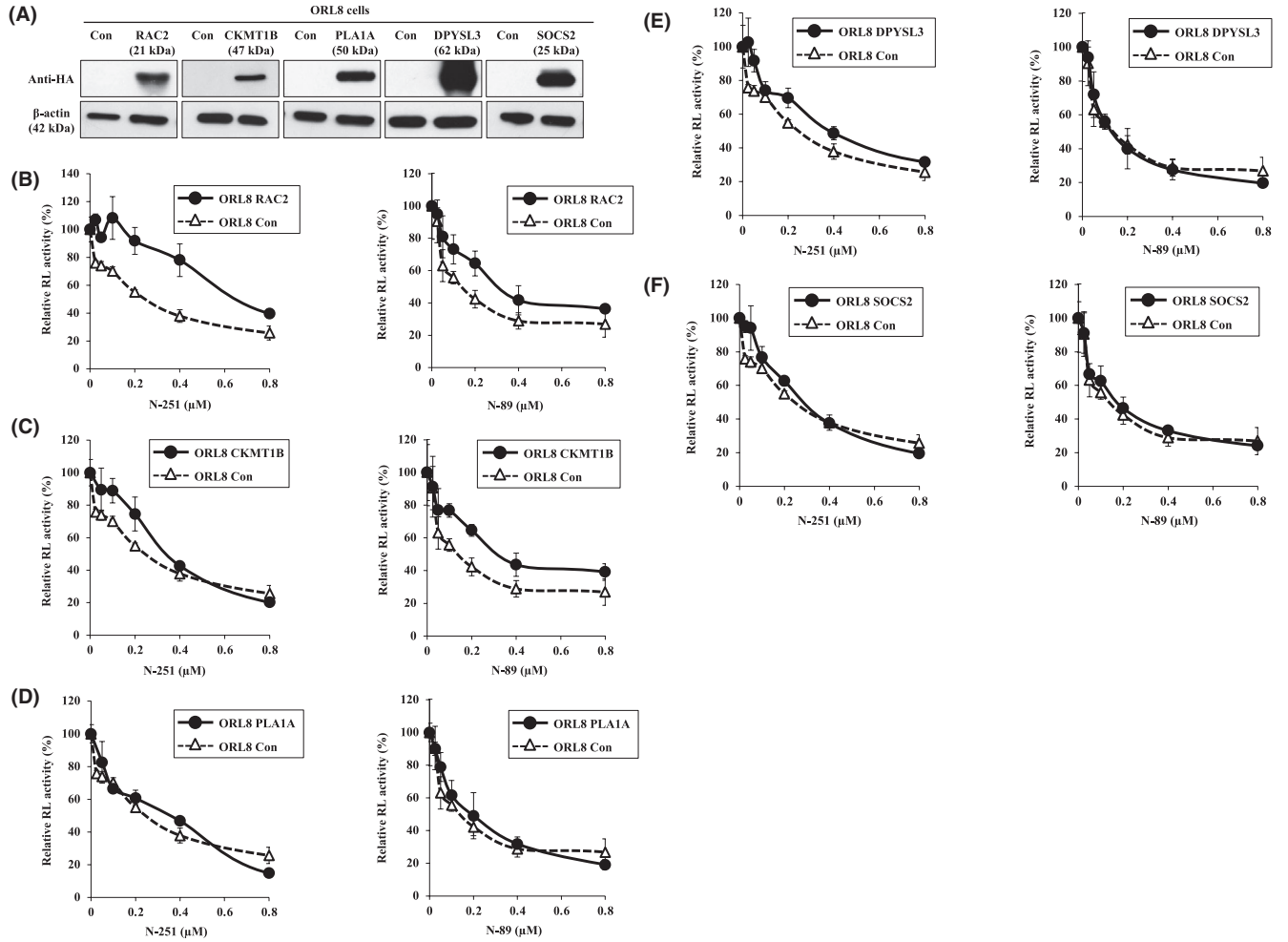


FIGURE 5 Overexpression of *RAC2* or *CKMT1B* contributed to the acquisition of an N-89(N-251)-resistant phenotype. A, Overexpression of each of five genes in the ORL8 cells. Western blot analysis was performed as described in Figure 2A. B–F, Evaluation of the anti-HCV activities of N-251 (left panel) and N-89 (right panel) in ORL8-cells overexpressing *RAC2* (B), *CKMT1B* (C), *PLA1A* (D), *DPYSL3* (E), and *SOCS2* (F). ORL8 cells infected with control retrovirus (ORL8 Con) were used as a control for the comparison. ORL8 Con (open triangles) and each gene overexpressing ORL8 cells (black circles) were treated with N-251 (left panel) or N-89 (right panel) for 72 h, and then an RL assay was performed as described in Figure 1D

TABLE 5 Anti-HCV activities of N-89(N-251) in ORL8 cells overexpressing each gene that potentially contributes to the acquisition of an N-89(N-251)-resistant phenotype

	N−251 (μM)		N−89 (μM)	
Cells	EC_{50}	Resistance (fold)	EC_{50}	Resistance (fold)
ORL8 Con	0.25	-	0.14	-
ORL8 RAC2	0.65	2.6	0.30	2.1
ORL8 CKMT1B	0.34	1.4	0.31	2.2
ORL8 PLA1A	0.35	1.4	0.19	1.4
ORL8 DPYSL3	0.36	1.4	0.13	0.93
ORL8 SOCS2	0.28	1.1	0.17	1.2

demonstrated that N-89(N-251) inhibited the RNA replication of an HCV replicon possessing regions NS3 to NS5B,[6] 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).[10] For the selection of the aa substitutions that may contribute to N-89(N-251) resistance, aa substitutions derived from known adaptive mutations[19,29-33] were first excluded from the detected aa substitutions, because adaptive mutations are known to appear naturally during the course of cell culture.[34-36] 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[7,34-36] were also excluded from the detected aa substitutions. However, more than 20 aa substitutions still remained as candidates even after these selections (Figure 6A).

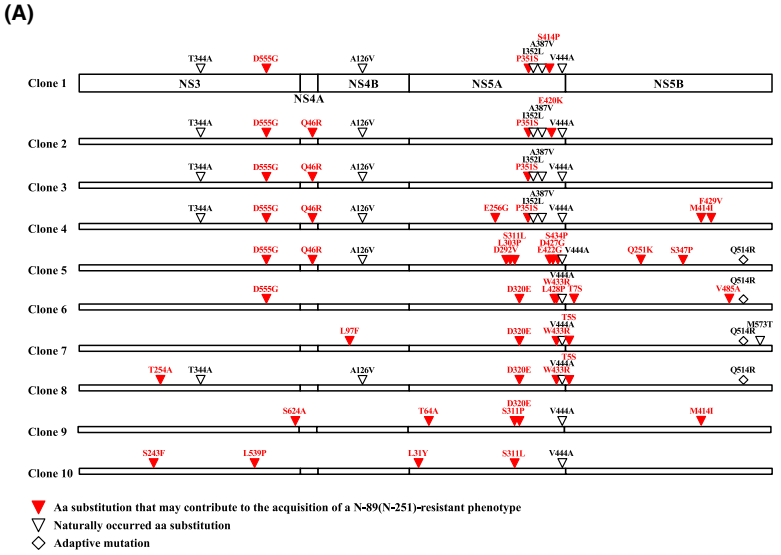
To narrow down these aa substitutions or targeted regions for N-89(N-251) resistance, we next decided to examine the N-89 sensitivity of HCV replicon RNA possessing the sequence (NS3 to NS5B regions) of each HCV cDNA clone. To perform this analysis, the NS3 to NS5B regions of each HCV cDNA clone were replaced with the NS3 to NS5B regions of HCV replicon RNA (OR/3-5B QR,KE) (Figure 6B),[19] and then the obtained RNA was introduced into ORL8 N-251rc cells by the electroporation method.[7] The N-89 sensitivity of HCV replicon RNA possessing the sequences of each HCV cDNA clone was examined by transient RNA replication reporter assay,[7] in comparison with the ORL8 N-251rc cells transfected with the HCV replicon RNA possessing the NS3 to NS5B regions from ORL8 cells (named wt). The results revealed that 7 (clones 4 to 10) of 10 clones exhibited some level of conversion into an N-89-resistant phenotype, although a significant conversion was observed only for clones 5, 6, and 7 (Figure 6C), suggesting that most of the HCV–RNAs obtained from ORL8 N-251r are able to increase the degree of resistance to N-89. We observed that the levels of HCV replicon RNAs were not significantly different among any of the clones except clones 3 and 5, which showed weakly higher or lower level of HCV replicon RNA than the wild type of replicon RNA (Figure 6D). Since clones 1 to 3 did not show an N-89-resistant phenotype (Figure 6C), D555G in NS3, Q46R in NS4A, and P351S in NS5A, which were detected in plural other clones, were excluded. Among the remaining aa substitutions, many aa substitutions in the C-terminal region of NS5A are notable. However, this region is known to be dispensable for HCV–RNA replication[37] and the mutations in this region are intensively accumulated during the long-term RNA replication.[34-36] Therefore, we assumed that aa substitutions in the NS5B region, but not in the NS5A region, are involved in N-89 resistance.

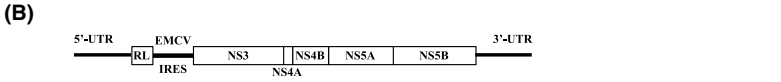
To check this assumption, using site-directed mutagenesis method,[7,19] we introduced one to three mutations detected in the NS5B region of clones 4 to 7 and 9 into HCV replicon RNA (OR/3-5B QR,KE).[19] Using the prepared HCV–RNA possessing mutation(s) in only the NS5B region, a transient RNA replication reporter assay for N-89 sensitivity was performed as described above. The results showed that the HCV replicon RNA possessing only a mutation (clone 9-derived M414I) significantly exhibited strong N-89 resistance, compared with that of the wt HCV replicon RNA (Figure 6E). However, the N-89 sensitivity of HCV replicon RNA possessing clone 4-derived M414I and F429 V mutations did not change at all (Figure 6E), suggesting that F429 V suppressed the effect of M414I. In addition, HCV replicon RNA possessing clone 6-derived T7S and V485A mutations and Q514R adaptive mutation also exhibited weak N-89 resistance, compared with that of the wt HCV replicon RNA (Figure 6E), suggesting that the T7S/V485A or T7S/V485A/Q514R mutations also weakly contributed to N-89 resistance. In any case, our results indicate that at least the M414I mutation alone is able to convert the cells into an N-89-resistant phenotype. It is noted that in the three-dimensional structure of the NS5B,[38] the position 414 is located in a thumb domain and is predicted to act as a primer buttress helix[39] (Figure 6F).

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

FIGURE 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-251r cells. Sequence analysis of the NS3 to NS5B regions of 10 clones was performed and the resulting sequence was compared with the HCV sequences obtained from parental ORL8 cells or long-term cultured Li23-derived cells. The selected and classified aa substitutions are shown. B, The structure of HCV replicon RNA (OR/3-5B QR,KE) used in the transient RNA replication reporter assay. C, Evaluation of the anti-HCV activity of N-89 in ORL8 N-251rc cells transfected with HCV replicon RNA possessing the NS3 to NS5B regions of HCV clones obtained from ORL8 N-251r cells. HCV replicon RNA (OR/3-5B QR,KE) was used as a control (wt). Each HCV replicon RNA-transfected cells were treated with 1 μM N-89 for 72 h, and then RT-qPCR analysis was performed as described in Figure 2C. The relative value (%) calculated for each time point when the level in non-treated cells was taken as 100% is presented. Data are the mean ± SD of triplicate assays. $*p < 0.05$ versus wt-transfected cells. D, The levels of HCV replicon RNAs in the cells used in C. RT-qPCR analysis was performed as described in Figure 2C. E, Evaluation of the anti-HCV activity of N-89 in ORL8 N-251rc cells transfected with HCV replicon RNA possessing one to three mutations causing aa substitution in the NS5B region of HCV clones obtained from ORL8 N-251r cells. HCV replicon RNA (OR/3-5B QR,KE) was used as a control (wt). Each HCV replicon RNA-transfected cells were treated with 1 μM N-89 for 72 h, and then RT-qPCR analysis was performed as described in Figure 2C. $*p < 0.05$, $**p < 0.01$ versus wt-transfected cells. F, Crystal structure of genotype 1b HCV NS5B RNA-dependent RNA polymerase (PDB code 1QUV) with the finger, palm, thumb, and C-terminal linker domains colored according to convention.[38] Location of M414 show the red color. The diagrams in this image were generated with UCSF Chimera v1.15


(A)
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
Clone 8
Clone 9
Clone 10
NS3
NS4A
NS4B
NS5A
NS5B
Aa substitution that may contribute to the acquisition of a N-89(N-251)-resistant phenotype
Naturally occurred aa substitution
Adaptive mutation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EMCV
IRES
NS3
NS4A
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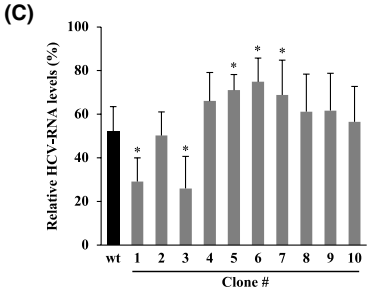
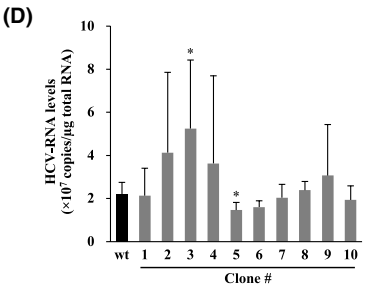
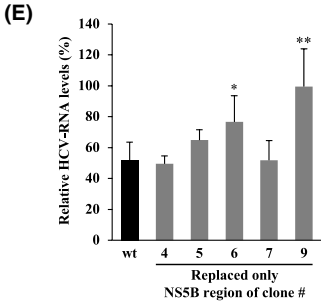
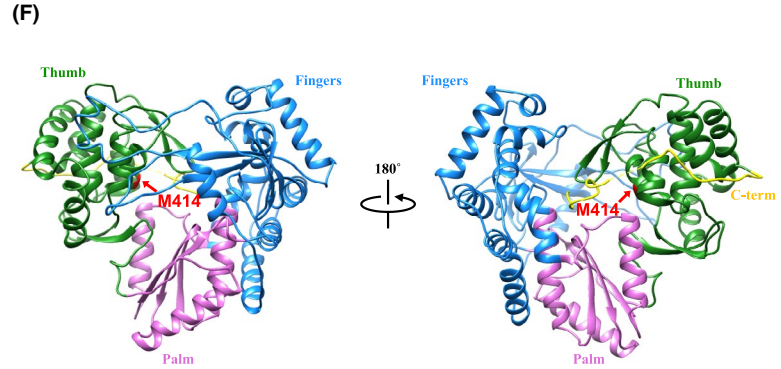
NS5A
NS5B
3'-UTR


(C)
Relative HCV-RNA levels (%)
wt
Clone #


(D)
HCV-RNA levels
(×10⁷ copies/μg total RNA)
wt
Clone #


(E)
Relative HCV-RNA levels (%)
wt
Replaced only
NS5B region of clone #


(F)
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Fingers
M414
Palm
180°
Fingers
Thumb
C-term
M414
Palm

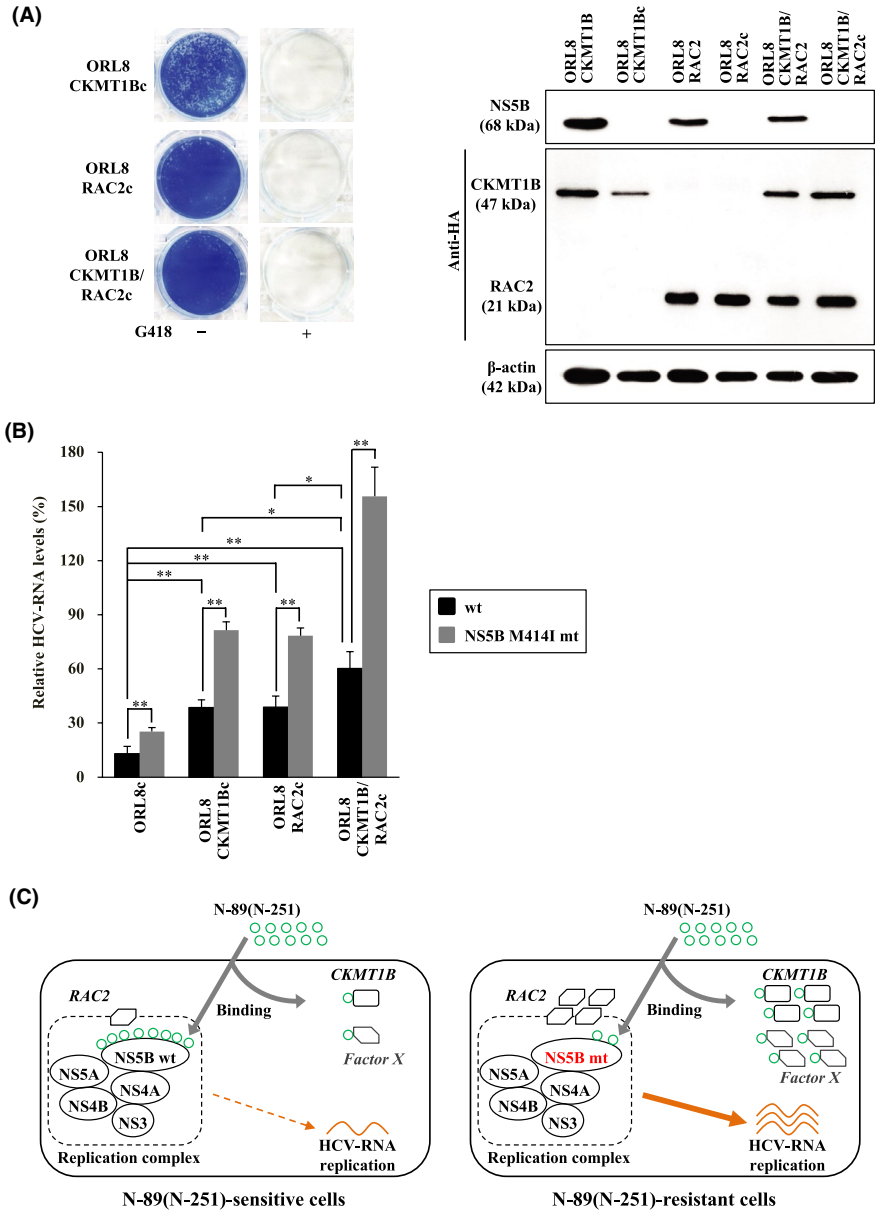

FIGURE 7 Two host factors (CKMT1B and RAC2) and a viral factor (M414I mutation) additively contributed to the acquisition of an N-89-resistant phenotype. A, ORL8 CKMT1B, ORL8 RAC2, and ORL8 CKMT1B/RAC2 cells were cured by treatment with IFN-γ. CBB staining (left panel) and Western blot analysis (right panel) were performed as described in Figure 2A. B, Evaluation of the anti-HCV activity of N-89 in ORL8c, ORL8 CKMT1Bc, ORL8 RAC2c, and ORL8 CKMT1B/RAC2c cells transfected with HCV replicon RNA possessing an M414I mutation. HCV replicon RNA (OR/3-5B QR,KE) was used as a control (wt). Each HCV replicon RNA-transfected cells were treated with 1 μM N-89 for 72 h, and then RT-qPCR analysis was performed as described in Figure 2C. $*p < 0.05$, $**p < 0.01$. C, Proposed action model of N-89(N-251) in N-89(N-251)-sensitive or -resistant cells

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 CKMT1B/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 M414I mutation independently and additively raised the degree of N-89 resistance in ORL8 CKMT1Bc, ORL8 RAC2c, or ORL8 CKMT1B/RAC2c cells (Figure 7B). It is noteworthy 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 weaken the activity of N-89(N-251) by catching N-89(N-251). Since there has been no 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 EC_{50} 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–RNA[37] 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).

ACKNOWLEDGMENTS

The authors thank Midori Shimizu, Fumie Ichimura, and Takashi Nakamura for their technical assistance. The authors also thank Dr. Hey-Sook Kim for providing N-89 and N-251. This research was supported by AMED under Grant Number JP18fk0210009 and JP20fk0210053.

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.

ORCID

Youki Ueda https://orcid.org/0000-0002-2627-8524
Hiromichi Dansako https://orcid.org/0000-0002-2353-3024
Shinya Satoh https://orcid.org/0000-0001-8683-1929

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How to cite this article: Gu W, Ueda Y, Dansako H, Satoh S, Kato N. Antiviral mechanism of preclinical antimalarial compounds possessing multiple antiviral activities. *FASEB BioAdvances*. 2021;3:356–373. https://doi.org/10.1096/fba.2020-00107