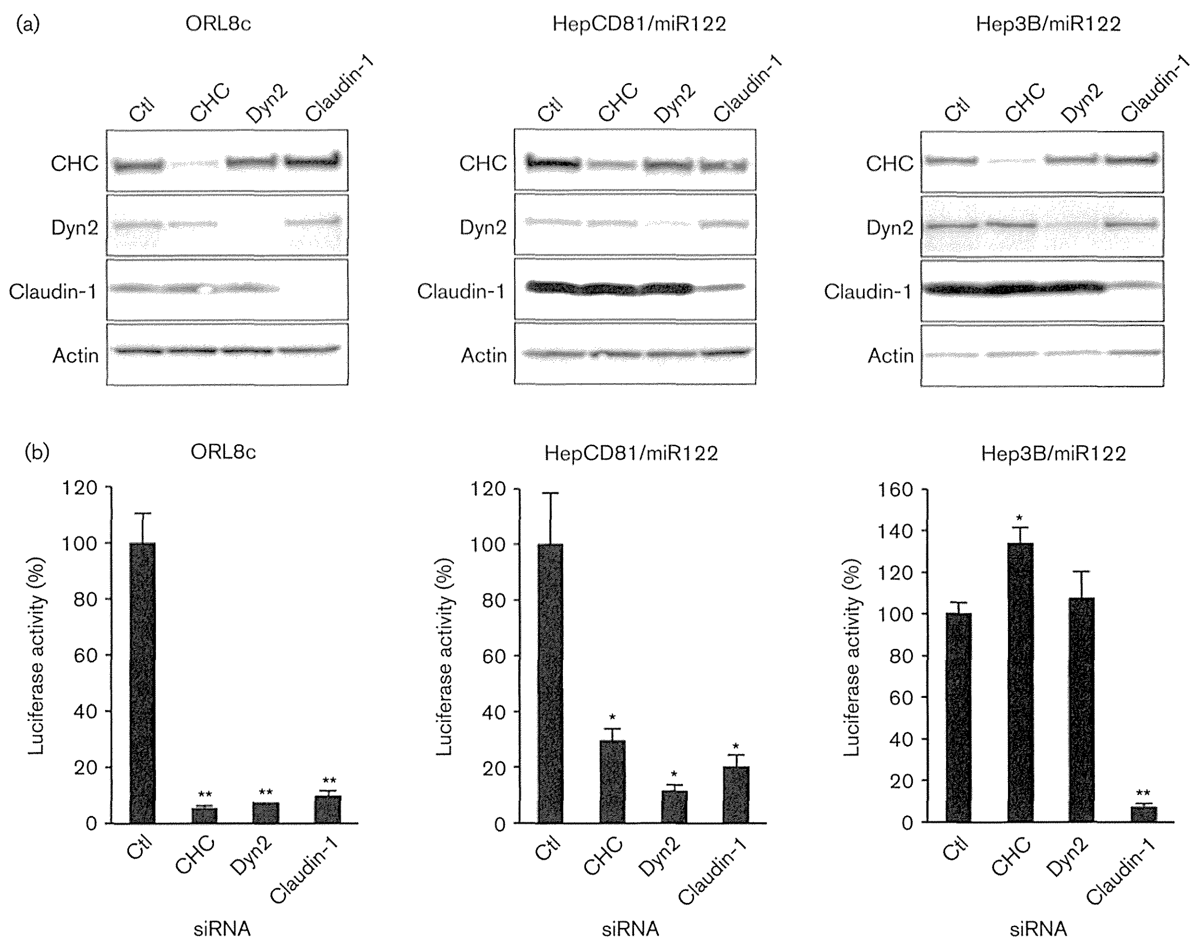

Fig. 6. Clathrin-dependent and -independent pathway of HCV entry in other HCV-permissive cells. The indicated cells were transfected with the indicated siRNAs and then infected with HCVtcp at 48 h post-transfection. (a) Specific knockdown of each protein was verified by immunoblotting. (b) Luciferase activity was determined at 72 h post-infection and expressed relative to the amount observed in the control (Ctl) siRNA transfection. Data represent the mean ± SD. Statistical differences between controls and each siRNA were evaluated using Student's *t*-test. *$P<0.05$, **$P<0.001$ versus control.

In summary, we identified an alternative clathrin- and dynamin-independent entry pathway for HCV in at least two independent cell lines, Huh7.5.1 and Hep3B/miR122 cells, in addition to the previously reported clathrin- and dynamin-dependent pathway. These findings provided clues for understanding the molecular mechanisms of the endocytosis pathway for HCV infection.

DISCUSSION

Many viruses have been shown to utilize a number of different endocytic pathways to productively infect their hosts. Clathrin-dependent endocytosis would appear to be the most commonly used, but it is increasingly clear that a number of clathrin-independent endocytosis pathways are also used by several different viruses (Mercer *et al.*, 2010). In the case of HCV, it has been reported that viral entry is mediated by clathrin-dependent endocytosis (Blanchard *et al.*, 2006; Codran *et al.*, 2006; Coller *et al.*, 2009; Meertens *et al.*, 2006; Trotard *et al.*, 2009). In these papers, HCVpp was used at least in part for analysis of HCV entry pathway. However, recent reports have revealed several different characteristics between HCVpp and HCVcc.

Viral entry has been addressed primarily by pharmacologic inhibitor studies, immunofluorescence and electron microscopy, by transfection with dominant-negative constructs, and more recently by siRNA knockdown. Analysis of endocytosis pathways using pharmacological inhibitors has raised concerns about specificity. For example, chlorpromazine, an inhibitor of clathrin-mediated endocytosis, has been shown to exert multiple side-effects on cell function as it targets numerous receptors and intracellular enzymes, and alters plasma membrane characteristics (Sieczkarski & Whittaker, 2002a). Methods for elucidating the viral endocytosis pathway by co-localization of virus particles with host factor also have limitations. Electron and

fluorescence microscopy, which require a high particle number, do not allow the differentiation of infectious and non-infectious particles. Infectious particles of HCV in the supernatant of infected cells appeared to represent only a small portion of secreted virus particles (Akazawa *et al.*, 2008) and it is unclear whether the viral particles observed by microscopy could lead to productive infection. Therefore, we utilized HCVtcp, which is useful for determining productive entry of the virus without reinfection, and a combination of siRNA knockdown and dominant-negative mutants for analysis of the productive route of infection. Although HCVcc is also utilized in analysis of productive entry, it cannot completely exclude the effects of reinfection by virus produced by infected cells. Reduction of HCVcc infection by knockdown of CHC and Dyn2 was moderate when compared with that of HCVtcp (Fig. 3c, d), thus suggesting slight effects due to reinfection in HCVcc.

The data presented here demonstrate for the first time to our knowledge that HCV is able to enter cells via dynamin-independent endocytosis in addition to the previously described classical clathrin- and dynamin-dependent pathway. First, knockdown of CHC and Dyn2 had no inhibitory effects on HCVtcp and HCVcc entry into Huh7.5.1 cells. Second, overexpression of dominant-negative Dyn2 had no inhibitory effects on HCVtcp in Huh7.5.1 cells. Finally, in addition to Huh7.5.1 cells, Hep3B/miR122 cells were also shown to be infected with HCV via clathrin- and dynamin-independent pathways. We further investigated the role of alternative minor routes of HCV entry into Huh7.5.1 cells; however, the productive endocytosis pathway could not be defined. It should be noted that inhibition of alternative endocytosis routes by siRNA led to an increase of luciferase activity (Figs 3c and 5a, c). This could be explained by the inhibition of a particular endocytosis pathway resulting in a compensatory increase in alternative endocytosis pathways (Damke *et al.*, 1995).

Although we confirmed an alternative endocytosis pathway for the productive entry of HCV, it is not clear why and how the two independent endocytosis pathways operate in different cell lines. SV40 can enter cells via caveolae-dependent (Norkin *et al.*, 2002; Pelkmans *et al.*, 2001) and -independent (Damm *et al.*, 2005) pathways. Influenza virus enters cells via clathrin-mediated endocytosis (Matlin *et al.*, 1981) in addition to non-clathrin-mediated, non-caveola-mediated internalization pathways (Sieczkarski & Whittaker, 2002b). Entry of dengue virus type 2 is clathrin-dependent in HeLa and C6/36 cells (Acosta *et al.*, 2008; Mosso *et al.*, 2008; van der Schaar *et al.*, 2008), and is clathrin-independent in Vero cells (Acosta *et al.*, 2009). Different receptor usage may determine the consequential route of entry. However, we did not observe any differences between Huh-7 and Huh7.5.1 cells in terms of knockdown effects of receptor candidate molecules on HCV infection, as shown in Fig. 1(c), although we cannot exclude the possibility that other undefined receptors are associated with viral entry. Huh7.5.1 cells were established by elimination of the HCV genome from replicon cells derived from Huh-7 cells (Blight *et al.*, 2002; Zhong *et al.*, 2005) and they exhibit more potent replication of HCV than the original Huh-7 cells. Further study showed that the increased permissiveness of cured cells results from a mutation in the retinoic acid-inducible gene I (Sumpter *et al.*, 2005), which impairs IFN signalling. In addition, it has been shown that cured cell lines express higher levels of miR122 than parental cells participating in the efficient propagation of HCVcc (Kambara *et al.*, 2012). As it is unclear whether these changes are the reason for a distinct endocytosis pathway, it will be of interest to explore these associations in further studies.

In conclusion, we confirmed an alternative clathrin-independent endocytosis pathway in HCV-permissive human hepatic-derived cells, in addition to the previously reported clathrin-dependent endocytosis pathway. This paper highlights the fact that clathrin- and dynamin-mediated endocytosis is the main route of HCV entry for Huh-7, HepCD81/miR122 and ORL8c cells, whilst clathrin and dynamin do not play a major role during the productive route of HCV infection in Huh7.5.1 and Hep3B/miR122 cells. Taken together, these studies suggest that different cell entry pathways for HCV infection may be utilized in different cell types, although further studies are necessary in order to understand this phenomenon.

METHODS

Cells. The human hepatocellular carcinoma cell lines Huh-7, Huh7.5.1, Hep3B/miR122 and HepG2/CD81, which overexpressed miR122 (Kambara *et al.*, 2012), were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industries) containing non-essential amino acids, penicillin (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10 % FBS. Li23-derived ORL8c cells (Kato *et al.*, 2009) were maintained in F12 medium and DMEM (1 : 1, v/v) supplemented with 1 % FBS, epidermal growth factor (50 ng ml^{-1}), insulin (10 μg ml^{-1}), hydrocortisone (0.36 μg ml^{-1}), transferrin (5 μg ml^{-1}), linoleic acid (5 μg ml^{-1}), selenium (20 ng ml^{-1}), prolactin (10 ng ml^{-1}), gentamicin (10 μg ml^{-1}), kanamycin monosulfate (0.2 mg ml^{-1}) and fungizone (0.5 μg ml^{-1}). All cell lines were cultured at 37 °C in a 5 % CO_2 incubator.

Preparation of viruses. HCVtcp and HCVcc derived from JFH-1 with adaptive mutations in E2 (N417S), p7 (N765D) and NS2 (Q1012R) were generated as described previously (Suzuki *et al.*, 2012). For HepCD81/miR122 and ORL8c cells, HCVtcp containing the *Gaussia* luciferase (GLuc) reporter gene were used. To do this, plasmid pHH/SGR-JFH1/GLuc/NS3m carrying the bicistronic subgenomic HCV replicon containing the GLuc reporter gene and the NS3 adaptive mutation was constructed by replacement of the firefly luciferase (FLuc) gene of pHH/SGR-Luc containing the NS3 mutation (N1586D) (Suzuki *et al.*, 2012) with the GLuc gene of pCMV-GLuc (NEB).

Plasmids. HA-tagged Dyn2, a dominant-negative Dyn2 (K44A) in which Lys44 was replaced with Ala, was cloned into pcDNA3.1 as described previously (Kataoka *et al.*, 2012).

Gene silencing by siRNA. siRNAs were purchased from Sigma-Aldrich and were introduced into the cells at a final concentration of

30 nM using Lipofectamine RNAiMAX (Invitrogen) in accordance with the manufacturer's instructions. Target sequences of the siRNAs were: occludin (5′-GCAAGAUCACUAUGAGACA-3′), SR-BI (5′-GAGCUU-UGGCCUUGGUCUA-3′), CD81 (5′-CUGUGAUCAUGAUCUUCGA-3′), CHC (5′-CUAGCUUUGCACAGUUUAA-3′), Dyn2 (5′-CCCUCA-AGGAGGCGCUCAA-3′), Cav1 (5′-CCCUAAACACCUCAACGAU-3′), flotillin-1 (5′-CCUAUGACAUCGAGGUCAA-3′), Arf6 (5′-CAGUU-CUUGGUAAAGUCCU-3′), CtBP1 (5′-GACUCGACGCUGUGCC-ACA-3′) and PAK1 (5′-GCAUCAAUUCCUGAAGAUU-3′). Target sequences of the siRNAs for claudin-1, PI4K and scrambled negative control were as described previously (Suzuki *et al.*, 2013).

Immunoblotting. Cells were washed with PBS and incubated with passive lysis buffer (Promega). Lysates were sonicated for 10 min and added to the same volume of 2× SDS-PAGE sample buffer. Protein samples were boiled for 10 min, separated by SDS-PAGE and then transferred to PVDF membranes (Merck Millipore). After blocking, membranes were probed with primary antibodies, followed by incubation with peroxidase-conjugated secondary antibody. Antigen–antibody complexes were visualized using an enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific) in accordance with the manufacturer's protocols.

Flow cytometry. Cultured cells detached by treatment with trypsin were incubated with anti-CD81 antibody or anti-mouse IgG antibody for 1 h at 4 °C. After being washed with PBS containing 0.1 % BSA, cells were incubated with an Alexa Fluor 488-conjugated anti-mouse secondary antibody (Invitrogen) for 1 h at 4 °C, washed repeatedly and resuspended in PBS. Analyses were performed using a FACSCalibur system (Becton Dickinson).

Reagents and antibodies. Bafilomycin A1 was obtained from Wako Pure Chemical Industries. Alexa Fluor 488-conjugated transferrin was obtained from Invitrogen. For immunoblotting, anti-SR-BI (NB400-104; Novus Biologicals), anti-occludin (71-1500; Invitrogen), anti-claudin-1 (51-9000; Invitrogen), anti-Dyn2 (ab3457; Abcam), anti-Cav1 (N-20; Santa Cruz Biotechnology), anti-flotillin (H-104; Santa Cruz Biotechnology), anti-Arf6 (ab77581; Abcam) and anti-PAK1 (2602; Cell Signaling Technology) rabbit polyclonal antibodies; anti-CD81 (JS-81; BD Biosciences), anti-β-actin (AC-15; Sigma-Aldrich), anti-CHC (23; BD Biosciences), anti-GRAF1 (SAB1400439; Sigma-Aldrich) and anti-glyceraldehyde 3-phosphate dehydrogenase (6C5; Merck Millipore) mouse mAb; and anti-CtBP1 goat polyclonal antibody (C-17; Santa Cruz Biotechnology) were used. For immunofluorescence staining, anti-CHC mAb (X22) and anti-HA rat polyclonal antibody (3F10) were obtained from Thermo Scientific and Roche Applied Science, respectively. Anti-NS5A antibody was a rabbit polyclonal antibody against synthetic peptides. Alexa Fluor 488- or 555-labelled secondary antibodies were obtained from Invitrogen.

DNA transfection. Cell monolayers were transfected with plasmid DNA using TransIT-LT1 transfection reagent (Mirus) in accordance with the manufacturer's instructions.

Treatment of cells with bafilomycin A1 and cell viability. Cells were preincubated with various concentrations of bafilomycin A1 for 60 min at 37 °C. Preincubated cells were then infected with HCVtcp. Cells treated with 0.1 % DMSO were used as controls. Cell viability was analysed by the Cell Titre-Glo Luminescent Cell Viability Assay (Promega).

Uptake of transferrin. Cells were grown on glass coverslips. After cells were transfected with HA-tagged Dyn2 expression plasmids, Alexa Fluor 488-conjugated transferrin at 20 μg ml^{-1} was added and incubated for 30 min. Cells were washed with PBS and fixed in 4 % paraformaldehyde.

Immunofluorescence analysis. Huh7.5.1 and Huh-7 cells were fixed with 4 % paraformaldehyde in PBS for 30 min, and were then blocked and permeabilized with 0.3 % Triton X-100 in a non-fat milk solution (Block Ace; Snow Brand Milk Products) for 60 min at room temperature. Samples were then incubated with anti-CHC, anti-Dyn2, anti-Cav1, anti-NS5A or anti-HA for 60 min at room temperature, washed three times with PBS, and then incubated with secondary antibodies for 60 min at room temperature. Finally, samples were washed three times with PBS, rinsed briefly in double-distilled H_2O and mounted with DAPI mounting medium. The signal was analysed using a Leica TCS SPE confocal microscope.

Luciferase assay. For quantification of FLuc activity in HCVtcp-infected cells, cells were lysed with passive lysis buffer (Promega) at 72 h post-infection. FLuc activity of the cells was determined using a luciferase assay system (Promega). For quantification of GLuc activity in supernatants of HCVtcp-infected cells, the *Renilla* Luciferase Assay System (Promega) was used. All luciferase assays were performed at least in triplicate.

Quantification of HCV core protein. HCV core protein was quantified using a highly sensitive enzyme immunoassay (Lumipulse G1200; Fujirebio) in accordance with the manufacturer's instructions.

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Synthesis and inhibitory activity on hepatitis C virus RNA replication of 4-(1,1,1,3,3,3-hexafluoro-2-hydroxy-2-propyl)aniline analogs

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ABSTRACT

Using our recently developed assay system for full-genome-length hepatitis C virus (HCV) RNA replication in human hepatoma-derived Li23 cells (ORL8), we identified 4-(1,1,1,3,3,3-hexafluoro-2-hydroxy-2-propyl)aniline analog **1a** as a novel HCV inhibitor. Structural modifications of **1a** provided a series of sulfonamides **7** with much more potent HCV RNA replication-inhibitory activity than ribavirin. Compound **7a** showed an additive anti-HCV effect in combination with standard anti-HCV therapy (IFN-α plus ribavirin). Since **7a** generated reactive oxygen species (ROS) in the ORL8 system and its anti-HCV activity was blocked by vitamin E, its anti-HCV activity may be mediated at least in part by ROS.

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Hepatitis C virus (HCV), an enveloped, single-stranded RNA virus, is a major cause of human hepatitis. It is estimated that ca. 150 million people worldwide are chronically infected with this virus, and more than 350,000 people die every year from hepatitis C-related liver disease.[1] Most infections become persistent and about 60% of cases progress to chronic liver disease, which in turn can lead to cirrhosis, hepatocellular carcinoma, and liver failure. Currently, no anti-HCV vaccine is available, and the standard treatment for chronic hepatitis C consists of pegylated interferon (IFN)-α in combination with the classical anti-HCV agent ribavirin. Recently, telaprevir, boceprevir and simeprevir, which are members of a new class of HCV NS3-4A serine-protease inhibitors, have been approved for hepatitis C treatment as combination therapy with pegylated IFN-α and ribavirin for patients with HCV genotype ***1a or 1b. Although these new therapies have increased cure rates for both previously untreated people and prior non-responders, there are still non-responders to these treatments. Also, serious adverse reactions such as skin reaction may require discontinuation of the combination therapy. Thus, new anti-HCV agents are still required, not only to extend the coverage of current therapy, but also because of the high mutation and replication rates of HCV.

The HCV genome consists of 9,600 bases encoding three structural and seven non-structural proteins.[2] So far, drug discovery programs for anti-HCV agents have focused on NS3 protease and NS5B polymerase as targets to block HCV replication. Conventionally, HCV replication assay has generally been conducted with subgenomic HCV replicon systems encoding minimum sequences for autonomous HCV replication, that is, HCV replicase proteins NS3 to NS5B, with firefly luciferase as the reporter gene.[3] However, we recently developed new screening systems to identify HCV inhibitors, namely a reporter gene assay system with *Renilla* luciferase for replication of genome-length HCV RNA in human hepatoma-derived HuH-7 cells (OR6)[4] and in human hepatoma-derived Li23 cells (ORL8).[5] Figure 1 shows a schematic representation of the gene organization in the genome-length HCV RNA in ORL8. These full-genomic systems are considered to be superior to the conventional sub-genomic systems, and are suitable for use in chemical-biological approaches to detect different types of HCV inhibitors due to the different genetic backgrounds of HuH-7 and Li23 cells.[6] By use of these assays, we identified *N*-{4-(1,1,1,3,3,3-hexafluoro-2-hydroxy-2-propyl)phenyl}-*N*-methylbenzamide (**1a**) as a novel HCV inhibitor (Fig. 2). Compound **1a** is a much more potent inhibitor of HCV RNA replication (EC_{50} 0.32 μM) than ribavirin (EC_{50} 8.7 μM). Interestingly, the ORL8 assay system

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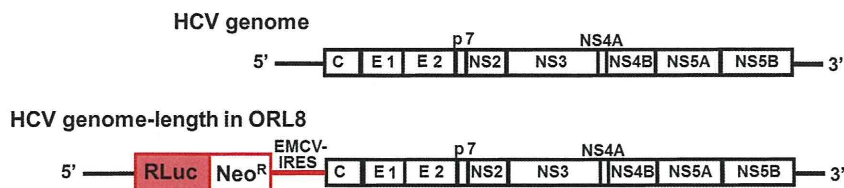


Figure 1. Schematic illustration of the gene organization in HCV genome-length in ORL8. *Abbreviations:* RLuc, *Renilla* luciferase gene; NeoR, neomycin phosphotransferase; EMCV-IRES, encephalomyocarditis virus internal ribosomal entry site.

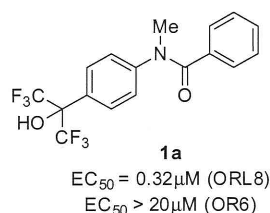


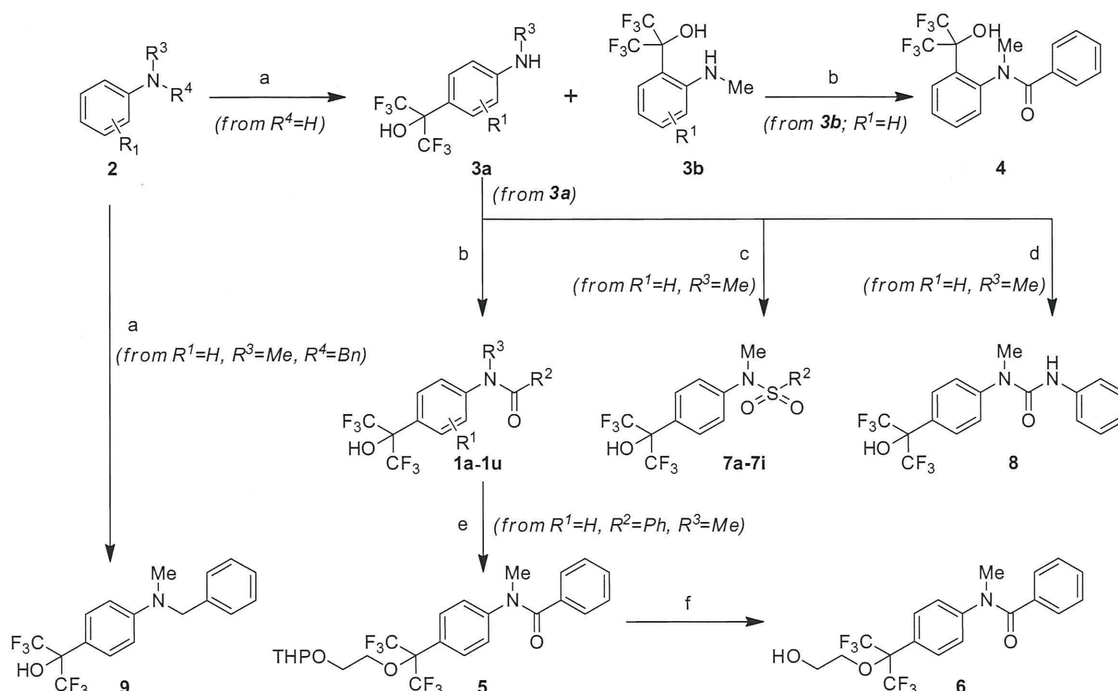
Figure 2. Structure of **1a** and HCV RNA replication-inhibitory activity in the ORL8 and OR6 assay systems.

was more sensitive to **1a** than the OR6 system. Herein, we describe the synthesis of **1a** and its derivatives modified at both phenyl rings, as well as at the hexafluoroisopropanol group and the amide moiety. The structure–activity relationship of these compounds for HCV RNA replication was examined, and the mechanism of action is discussed.

The synthetic procedures are outlined in Scheme 1. Briefly, condensation of (1,1,1,3,3,3-hexafluoro-2-hydroxy-2-propyl)-aniline **3a** with acyl chloride, sulfonyl chloride or isocyanate provided amides (**1a–1u**), sulfonamides (**7a–7i**) or urea (**8**), respectively. Compound **3a** was obtained as the major product by alkylation of aniline **2** with hexafluoroacetone trihydrate under acidic conditions.[7] Compound **3b** was also formed as a minor product, so the regioisomer of the hexafluoroisopropanol group (**4**) was synthesized from **3b** by benzoylation. Incorporation of substituents on phenyl rings and phenyl ring replacement were achieved by using the corresponding commercially available aniline **2**, acyl chloride or sulfonyl chloride. 1,1,1,3,3,3-Hexafluoro-2-(2-hydroxyethoxy)propyl analog **6** was synthesized from THP-protected **5**, which was obtained by alkylation of **1a** with commercially available 2-(2-bromoethoxy)tetrahydro-2*H*-pyran. The aminomethylene analog **9** was synthesized by direct incorporation of a hexafluoroisopropanol moiety into *N*-benzyl-*N*-methylaniline.

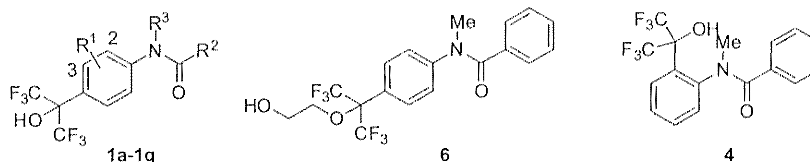
All synthetic analogs were assayed for anti-HCV RNA replication activity in the ORL8 system and EC_{50} values were determined (Tables 1 and 2). The values of 50% cytotoxic concentration (CC_{50}) for Li23 cells were determined by conventional WST-1 assay, and the values of selectivity index (SI), that is CC_{50}/EC_{50}, were calculated. All assays were conducted in triplicate.

The results of modifications at both phenyl rings and at the hexafluoroisopropanol group are summarized in Table 1. The incorporation of Me on the left phenyl ring (**1b**, **1c**) reduced anti-HCV RNA replication activity, and the 3-Me analog was completely inactive. Methylation of the right phenyl ring (**1d–1f**) also decreased anti-HCV activity. Among compounds with replacement of the right phenyl ring (**1g–1q**), alkyl analogs showed moderate activity. Extension of the alkyl chain increased the activity (**1g–1i**) and the cyclohexyl analog **1j** was as potent as the parent **1a**, with an adequate SI value. Bulky 1-adamantyl analog **1k** was a



Scheme 1. Synthesis of 4-(1,1,1,3,3,3-hexafluoro-2-hydroxy-2-propyl)aniline analogs. Reagents and conditions: (a) hexafluoro-acetone trihydrate, *p*TsOH hydrate, toluene, reflux, 71–93%; (b) R^2-COCl, Et_3N, toluene or $CHCl_3$, 22–93%; (c) R^2-SO_2Cl, Et_3N, CH_2Cl_2, quant.; (d) PhNCO, toluene, quant.; (e) $THPOCH_2CH_2Br$, Cs_2CO_3, DMF, 79%; (f) *p*TsOH hydrate, *i*PrOH, H_2O, quant.

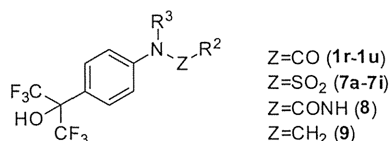
Table 1
Values of HCV RNA replication-inhibitory activity, cytotoxicity and safety index of **1a–1q**, **4** and **6** in ORL8 assay



Compd no	R[1]	R[2]	R[3]	EC_{50} (μM)	CC_{50} (μM)	SI[a]
1a	H	Ph	Me	0.32	65	203
1b	2-Me	Ph	Me	1.7	9.0	5.3
1c	3-Me	Ph	Me	>100	87	ND[b]
1d	H	2-Me-Ph	Me	17	38	2.2
1e	H	3-Me-Ph	Me	5.8	20	3.4
1f	H	4-Me-Ph	Me	10	26	2.6
1g	H	Me	Me	14	40	2.9
1h	H	nPr	Me	3.8	>100	ND[b]
1i	H	nBu	Me	2.3	26	11
1j	H	cHex	Me	0.23	20	87
1k	H	1-Adamantyl	Me	1.0	17	17
1l	H	CH_2Ph	Me	>100	27	ND[b]
1m	H	2-pyridyl	Me	>100	>100	ND[b]
1n	H	3-pyridyl	Me	45	63	1.4
1o	H	4-pyridyl	Me	8.2	40	4.9
1p	H	2-thienyl	Me	2.6	33	13
1q	H	3-thienyl	Me	17	44	2.6
6				11	>20	ND[b]
4				>20	>20	ND[b]

[a] Selectivity index (CC_{50}/EC_{50}).
[b] Not determined.

Table 2
Values of HCV RNA replication-inhibitory activity, cytotoxicity and safety index of **1r–1u**, **7a–7i**, **8** and **9** in ORL8 assay



Compd no	R[2]	R[3]	EC_{50} (μM)	CC_{50} (μM)	SI[a]
1r	Ph	H	4.1	12	2.9
1s	Ph	Et	0.78	19	24
1t	Ph	nPr	3.5	15	4.3
1u	Ph	Bn	2.9	7.5	2.6
7a	Ph	Me	0.19	16	84
7b	2-Cl-Ph	Me	0.17	15	88
7c	2-Br-Ph	Me	0.50	9.1	18
7d	2-Me-Ph	Me	0.36	12	33
7e	3-F-Ph	Me	0.36	19	53
7f	3-Me-Ph	Me	0.35	11	31
7g	4-Me-Ph	Me	0.44	15	34
7h	3-MeO-Ph	Me	2.1	13	6.2
7i	4-MeO-Ph	Me	1.4	7.9	5.6
8	Ph	Me	5.7	>20	ND[b]
9	Ph	Me	3.0	13	4.3

[a] Selectivity index (CC_{50}/EC_{50}).
[b] Not determined.

moderately active HCV inhibitor, while the benzyl analog **1l** was inactive. This result suggests the importance of the orientation of the hydrophobic moiety (vide infra). Suggestively, 2-pyridyl analog **1m**, whose hydrophilic N atom is located at the most proximal position to the amide moiety, was completely inactive. The conformation of the two aromatic rings could differ from those of other pyridyl analogs (**1n–1o**) at low pH values, such as that in endosomes of cells (vide infra).[8] Bioisosteric replacement of the phenyl ring with thiophene (**1p–1q**) decreased the activity.

As for hexafluoroisopropanol modifications, 2-hydroxyethoxy analog **6** was a weak HCV inhibitor. This result suggests that the acidity of OH might be important for anti-HCV activity (calculated pK_a[9] of OH group: 7.7 for **1a**, 14.4 for **6**, respectively). The position of the hexafluoroisopropanol group appears to be critical, since regioisomer **4** was inactive.

Inhibitory activities of the amide-modified analogs on HCV RNA replication are listed in Table 2. Among them, sulfonamide analog **7a** showed potent anti-HCV activity. In contrast to the amide series, substituents on the right phenyl ring (**7b–7g**) had little detrimental effect, except for OMe analogs (**7h–7i**), which showed >10-fold weaker activity and low SI values. Both deletion of the Me group on the amide moiety (**1r**) and extension of the alkyl group (**1s–1u**) decreased the anti-HCV activity. Insertion of NH (urea analog **8**) and carba replacement of the amide carbonyl (aminomethylene analog **9**) also reduced the anti-HCV activity by about 10-fold compared with **1a**. It is generally accepted that benzanilide takes *transoid* conformation, whereas *N*-methylated benzanilide uniquely takes *cisoid* conformation in aqueous solution.[10] We speculate that adequate proximity of the right phenyl ring (or hydrophobic group such as cHex) to the left phenyl ring, namely *cisoid* conformation of amide, is favorable for potent inhibition of HCV RNA replication. This would be consistent with the inactivity of the 2-pyridyl analog (**1m**), which would adopt *transoid* conformation at low pH.[8] Also, this is also supported by the following speculations; the moderate inhibitor urea **8** takes similar conformation of **1a**, namely *cisoid* (at –NMeCO–)-*transoid* (at –CONH–) conformation,[11] whereas the inactive benzyl analog **1l** might take complete cofacial conformation of the two phenyl ring due to the π–π stacking interaction.

Next, the effect of adding **7a** to current standard HCV therapy (IFN-α plus ribavirin) was evaluated (Fig. 3). In our ORL8 assay system, HCV RNA replication was dose-dependently reduced both by IFN-α alone and by IFN-α plus ribavirin. Addition of **7a** dose-dependently enhanced the inhibition of HCV RNA replication by these agents. Also, addition of **7a** to IFN-α plus ribavirin was more

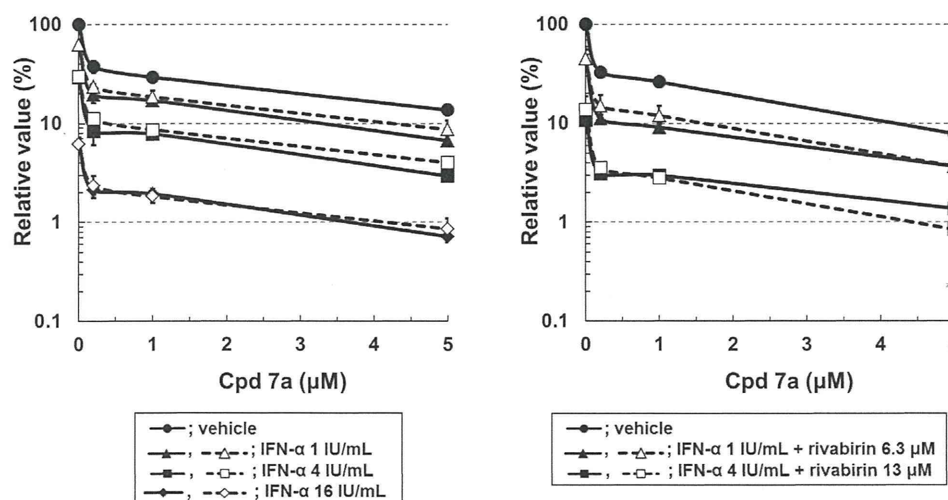


Figure 3. Additive effect of **7a** on HCV RNA replication in combination with IFN-α alone (left) and with IFN-α + ribavirin (right). (Left) circle: vehicle, triangle: IFN-α 1 IU/mL (IU: international unit), box: IFN-α 4 IU/mL), diamond: IFN-α 16 IU/mL. (Right) circle: vehicle, triangle: IFN-α 1 IU/mL + ribavirin 6.3 μM, box: IFN-α 4 IU/mL + ribavirin 13 μM. Open symbols in the broken lines show the values expected as an additive anti-HCV effect, and closed symbols in the solid lines show the values obtained by the ORL8 assay. Assays were conducted in triplicate.

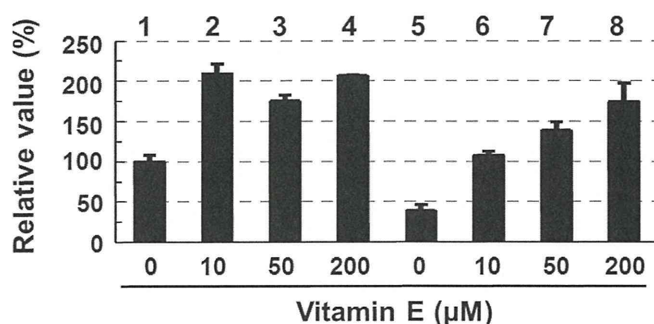


Figure 4. Effect of vitamin E on anti-HCV activity of **7a**. 1–4: in the absence of **7a**, 5–8: in the presence of 5 μM **7a** (60% inhibitory concentration for HCV RNA replication). Assays were conducted in triplicate.

effective than addition to IFN-α alone at the same concentration of IFN-α. These observations indicate that combination of **7a** with the current standard therapy might improve the cure rate of HCV infection.

Finally, the effect of vitamin E on the anti-HCV activity of **7a** was evaluated. As we previously reported, vitamin E strongly enhances HCV RNA replication.[12] Here, we examined the effect of vitamin E at various concentrations on HCV RNA replication in the presence of **7a** at the 60% inhibitory concentration level. As shown in Figure 4, vitamin E enhanced HCV RNA replication and dose-dependently blocked the anti-HCV activity of **7a**. We speculate that the 4-(1,1,1,3,3,3-hexafluoro-2-hydroxy-2-propyl)aniline analogs synthesized in this study have nuclear receptor-modulating activity, since structurally similar T0901317 is known to be a modulator of multiple nuclear receptors (NRs), including liver X receptor (LXR),[13] retinoic acid receptor-related orphan receptor gamma (RORγ)[14] and farnesoid X receptor (FXR).[15] Modulation of NRs produces reactive oxygen species (ROS) in various cells.[16,17] Indeed, **7a** dose-dependently produced ROS in the ORL8 assay system as determined by FACS analysis (data not shown). Therefore, we speculated that the mechanism of the anti-HCV activity of **7a** involves inhibition of HCV RNA replication by ROS produced via modulation of unknown NR(s). Indeed, scavenging of ROS by the antioxidant vitamin E blocked the anti-HCV activity of **7a**. Detailed studies on the mechanism of action of **7a** are ongoing.

In conclusion, *N*-{4-(1,1,1,3,3,3-hexafluoro-2-hydroxy-2-propyl)phenyl}-*N*-methylbenzamide (**1a**) was identified as a novel HCV inhibitor by use of our recently developed assay system for full-genome-length HCV RNA replication in Li23 cells (ORL8). Structural modifications of **1a** provided a series of sulfonamides **7** that showed potent inhibition of HCV RNA replication. Compound **7a** showed an additive anti-HCV effect in combination with standard HCV therapy (IFN-α plus ribavirin). The anti-HCV action of **7a** may be mediated by ROS production, since it was abrogated by vitamin E.

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IPS-1 Is Essential for Type III IFN Production by Hepatocytes and Dendritic Cells in Response to Hepatitis C Virus Infection

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Hepatitis C virus (HCV) is a major cause of liver disease. The innate immune system is essential for controlling HCV replication, and HCV is recognized by RIG-I and TLR3, which evoke innate immune responses through IPS-1 and TICAM-1 adaptor molecules, respectively. IL-28B is a type III IFN, and genetic polymorphisms upstream of its gene are strongly associated with the efficacy of polyethylene glycol–IFN and ribavirin therapy. As seen with type I IFNs, type III IFNs induce antiviral responses to HCV. Recent studies established the essential role of TLR3–TICAM-1 pathway in type III IFN production in response to HCV infection. Contrary to previous studies, we revealed an essential role of IPS-1 in type III IFN production in response to HCV. First, using IPS-1 knockout mice, we revealed that IPS-1 was essential for type III IFN production by mouse hepatocytes and CD8$^+$ dendritic cells (DCs) in response to cytoplasmic HCV RNA. Second, we demonstrated that type III IFN induced RIG-I but not TLR3 expression in CD8$^+$ DCs and augmented type III IFN production in response to cytoplasmic HCV RNA. Moreover, we showed that type III IFN induced cytoplasmic antiviral protein expression in DCs and hepatocytes but failed to promote DC-mediated NK cell activation or cross-priming. Our study indicated that IPS-1–dependent pathway plays a crucial role in type III IFN production by CD8$^+$ DCs and hepatocytes in response to HCV, leading to cytoplasmic antiviral protein expressions. *The Journal of Immunology,* **2014, 192: 2770–2777.**

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Hepatitis C virus (HCV) is a major cause of chronic liver disease (1). The 3′ untranslated region (UTR) of the HCV genome is recognized by a cytoplasmic viral RNA sensor RIG-I (2). HCV RNA induces RIG-I–dependent type I IFN production to promote hepatic immune responses in vivo (2). RIG-I is a member of RIG-I–like receptors (RLRs), which include MDA5 and LGP2. RLRs trigger signal that induces type I IFN and other inflammatory cytokines through the IPS-1 adaptor molecule (3). RLRs are localized in the cytoplasm and recognize cytoplasmic dsRNAs. Another pattern recognition receptor, TLR3, recognizes dsRNAs within early endosomes or on cell surfaces (4). Human monocyte-derived dendritic cells (DCs) require TLR3 to recognize HCV RNA in vitro (5), and TLR3 induces type I IFN production through the TICAM-1 adaptor, also called Toll/IL-1R domain-containing adapter inducing IFN-β (6, 7).

IL-28B is a type III IFN (also called IFN-λ), which includes IL-28A (IFN-λ2) and IL-29 (IFN-λ1) (8). Type III IFNs interacts with heterodimeric receptors that consist of IL-10Rβ and IL-28Rα subunits (8). Polymorphisms upstream of the IL-28B (IFN-λ3) gene are significantly associated with the responses to polyethylene glycol–IFN and ribavirin in patients with chronic genotype 1 HCV infections (9–12). As seen with type I IFNs, type III IFNs have antiviral activities against HCV (13). Type I IFNs induce the expression of IFN-inducible genes, which have antiviral activities, and can promote cross-priming and NK cell activation (14). However, the roles of type III IFN in cross-priming and NK cell activation are largely unknown, and the functional differences between type I and III IFN are uncertain.

Mouse CD8$^+$ DCs and its human counterpart BDCA3$^+$ DCs are the major producers of type III IFNs in response to polyI:C (15). CD8$^+$ DCs highly express TLR3 and have strong cross-priming capability (16). A recent study showed that TLR3 was important for type III IFN production by BDCA3$^+$ DCs in response to cell-cultured HCV (17). RIG-I efficiently recognizes the 3′ UTR of the HCV RNA genome, and, thus, RIG-I adaptor IPS-1 is essential for type I IFN production (2). However, the role of an IPS-1–dependent pathway in type III IFN production in vivo has been underestimated. In this study, we investigated the role of an IPS-1–dependent pathway in type III IFN production in vivo and in vitro using IPS-1 knockout (KO) mice and established an essential role of IPS-1 in type III IFN production in response to HCV RNA. Our study indicated that not only TICAM-1 but also IPS-1 are essential for type III IFN production in response to HCV.

Materials and Methods

Mice

All mice were backcrossed with C57BL/6 mice more than seven times before use. The generation of TICAM-1 and IPS-1 KO mice was described

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM-DC, bone marrow–derived dendritic cell; BM-Mf, bone marrow–derived macrophage; DC, dendritic cell; HCV, hepatitis C virus; KO, knockout; Mf, macrophage; Oc, O cured; RLR, RIG-I–like receptor; UTR, untranslated region.

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