In this report, we show that dicer-generated siRNAs from long dsRNA can silence the replication of HCV RNA of different genotypes. Furthermore, we employed the modified long hairpin-RNA (hRNA) expression system to inhibit HCV replication and to avoid triggering the IFN response, which is normally caused by dsRNA. We demonstrated that intracellular-diced dsRNA can be used in mammalian cells to silence HCV RNA and, therefore, that long dsRNA-mediated RNAi could be useful as a therapeutic agent for natural viral infection by HCV.

Results

Synthetic small interfering RNA inhibits hepatitis C virus replication in HuH-7 cells containing replicating hepatitis C virus RNA

As siRNA-mediated RNAi is strictly sequence specific, an siRNA targeting site was selected in regions conserved among the various HCV genotypes. Of the HCV genome sequences, the 5′-UTR and the 3′X region are the most highly conserved.[19] Therefore, we selected six sites in the 5′-UTR or core coding regions (A–F) and three sites in the 3′X regions (G–I) (see Materials and methods). HuH-7 cells carrying the HCV replicon were established as described.[20] We also modified the replicon RNA derived from the HCV genotype 1b clone (GenBank accession number AY045702) by substituting the *neo*^{r} gene with the firefly luciferase gene fused to foot-and-mouth disease virus (FMDV) 2A and the *neo*^{r} gene (named the R6FLR-N replicon). This modification enables the sensitive and precise quantification of HCV replication levels using a luciferase assay.

To examine the ability of siRNAs to inhibit HCV replication, the nine synthetic siRNAs were transfected into R6FLR-N replicon cells (Figure 1a, left). Of the siRNAs, siE (nucleotides (nt) 325–344) was the most effective and it dose-dependently inhibited HCV replication (Figure 1a, right). Moreover, continuous transfection with siE but not the negative control p53m siRNA caused a gradual decrease in the HCV replicon titer up to the 23rd day (Figure 1b). Using Northern blot analysis, we confirmed that the effects of siRNAs on the luciferase activity are associated with siRNA-directed degradation of the HCV replicon RNA (data not shown). These results indicated that siE was the most potent siRNA for inhibiting HCV replication of the selected siRNA sites.

Effect of ex-vivo *dicer-generated small interfering RNAs from long double-stranded RNA*

We found that shifting the siRNAs 5′ or 3′ from the siE target position reduced the efficacy of siRNA-mediated RNAi (Figure 2a). Therefore, to overcome site specificity of the selected siRNAs, we prepared *ex-vivo* recombinant human dicer (rhDicer)-generated siRNAs (d-siRNAs) from long dsRNAs (Figure 2b).[21] R6FLR-N replicon cells were transfected with d-siRNAs targeting the HCV genome or p53 mRNA (negative control). Luciferase reporter assays indicated that d-siRNAs generated from the 5′-UTR of HCR6 sequences (D5-357, D5-197, and D5-50) silenced the HCV RNA more efficiently than siE. In contrast, the d-siRNAs generated from the 3′-UTR of the HCR6 sequences were less effective than siE. These

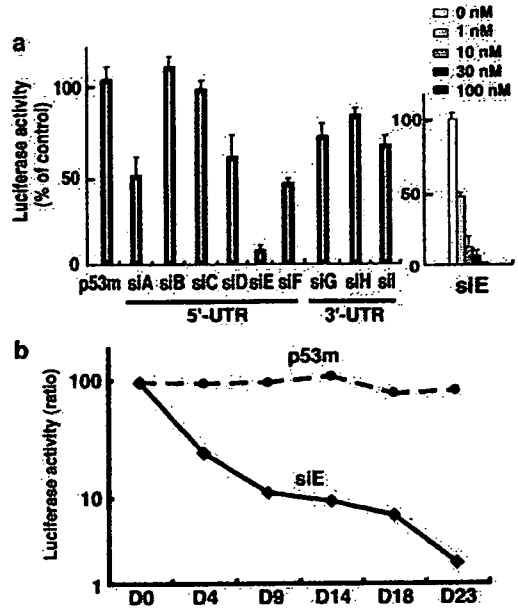

Figure 1 Effect of siRNA on HCV replication. (a) Inhibition of the HCV replicon by siRNAs in R6FLR-N replicon cells. Luciferase activity was measured 24 h after transfection using the Trans IT TKO reagent. Data represent means ± s.d. ($n = 3$) compared with mock-transfected cells. siA to siF, siRNA in the 5′-UTR or core cording regions; siG to siI, siRNA in the 3′-UTR; p53 m, negative control siRNA. (b) Long-term effect of siE. The R6FLR-N replicon cells were transfected with siRNAs every 4 days. Luciferase activity was measured on the indicated days.

results indicated that d-siRNAs generated from 5′-UTR containing the siE sequences, especially those generated from 197-bp dsRNAs, were more effective than the synthetic siE.

Dicer-generated siRNAs generated from 197-bp double-stranded RNA overcome hepatitis C virus genotype variation

Genotype 1b-derived d-siRNAs generated from the conserved sequence motifs within the NS5B sequence do not block the replication of HCV genotypes 1a and 2a.[14] To examine whether our selected d-siRNAs can overcome HCV genotype variation, we transfected genotype 2a-specific d-siRNAs into R6FLR-N replicon cells, which harbor the genotype 1b replicon. As shown in Figures 3a and b, the genotype 2a-derived d-siRNAs generated from 197-bp dsRNA efficiently inhibited genotype 1b replication, even though genotypes 2a and 1b differ by 15 bases within the 197-bp dsRNA sequences (sequence homology = 92%). In contrast, genotype 2a-derived siE, which harbors a single mutation at position 18 of the sense strand (sequence homology = 95%), showed a weak silencing activity against genotype 1b. These results demonstrated that d-siRNAs generated from the 197-bp dsRNA were highly effective for RNAi and could overcome HCV genotype variation.

Long double-stranded RNA transfection into HuH-7 replicon cells induces target-specific silencing

Dicer is a large multi-domain protein present in all eukaryotes.[22] Recently, Kim *et al*.[23] reported that syn-

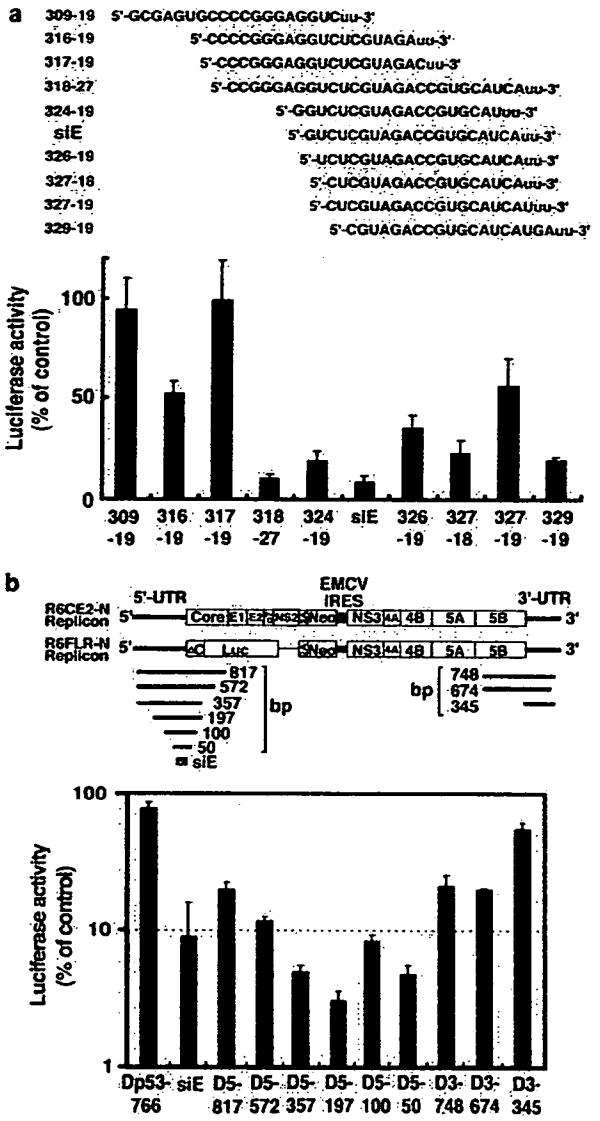

Figure 2 Small interfering RNAs cleaved by rhDicer from long dsRNA. (a) Effect of the positional variations in the siE region. R6FLR-N replicon cells were transfected using Lipofectamine 2000 with siRNAs in which the target position was shifted towards either the 5′- or 3′-end of the siE region. Luciferase activity assay measured 48 h after transfection with 1 nM siRNAs. Data represent means±s.d. compared with mock-transfected cells ($n=5$). (b) Upper panel, schematic representation of the long dsRNAs used for targeting different sites in the HCV genome RNA; lower panel, effect of d-siRNAs. The d-siRNAs were generated from the long dsRNAs by cleavage with rhDicer. R6FLR-N cells were transfected with d-siRNAs. Luciferase activity was measured after 48 h. Data represent means±s.d. compared with mock-transfected cells ($n=5$). Dp53-766, which targeted p53 mRNA (766 bp), was used as a negative control.

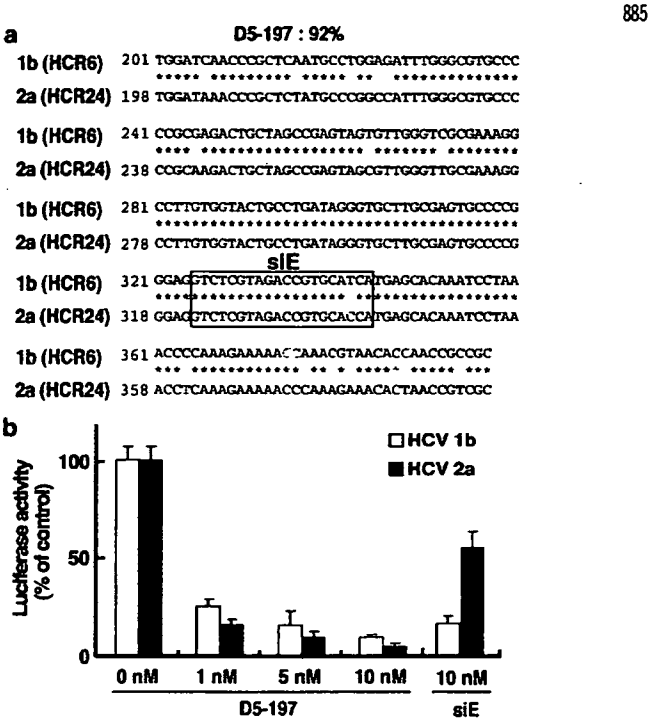

Figure 3 Dicer-generated siRNAs directed at the HCV genotype 2a can cause silencing of genotype 1b RNA. (a) The sequence homology between genotypes 1b and 2a was 92% within the 197-bp region (182/197 nt) and 95% within the 20-bp siE region (19/20 nt). (b) R6FLR-N cells harboring the genotype 1b HCV replicon RNA were transfected with the d-siRNAs generated from a 197-bp dsRNA directed at HCV genotype 2a (HCR24; accession number AY746460). Data represent means±s.d. compared with mock-transfected cells ($n=5$).

thetic RNA duplexes 25–30 nt in length are substrates of the dicer endonuclease, directly linking the production of siRNAs to incorporation in the RNA-induced silencing complex. We also expected that intracellular dsRNA duplexes longer than 50 nt would be recognized by dicer and thus induce RNAi. Therefore, we directly transfected long dsRNA into R6CE2-N replicon cells, which harbor the core to NS2 portion of the HCV genome (Figure 2b). The same amount of dsRNA was transfected into replicon cells, and the replicon copy number was determined by quantitative real-time detection (RTD)-polymerase chain reaction (PCR).[24] We found that, except for the 817-bp dsRNA, the long dsRNAs targeting sites in the HCV genome reduced the HCV RNA copy number. In contrast, an unrelated dsRNA targeting a site in endogenous p53 mRNA had no effect (Figure 4a). A luciferase assay in R6FLR-N replicon cells showed similar results for HCV-specific silencing (data not shown). On the other hand, immunoblot analysis with antibodies against p53 showed that p53-specific long dsRNA suppressed the level of p53 protein, whereas HCV-specific dsRNA had no effect on p53 expression (Figure 4b). These results indicated that in HuH-7 replicon cells, direct transfection of long dsRNA can specifically produce RNAi against HCV and reduce endogenous p53 expression.

Effect of long double-stranded RNA on the intracellular interferon response and cell death in HepG2 cells

In mammalian cells, Toll-like receptor (TLR) 3[25,26] recognizes dsRNA duplexes longer than 30 nt. This binding induces a type I IFN response, resulting in cell

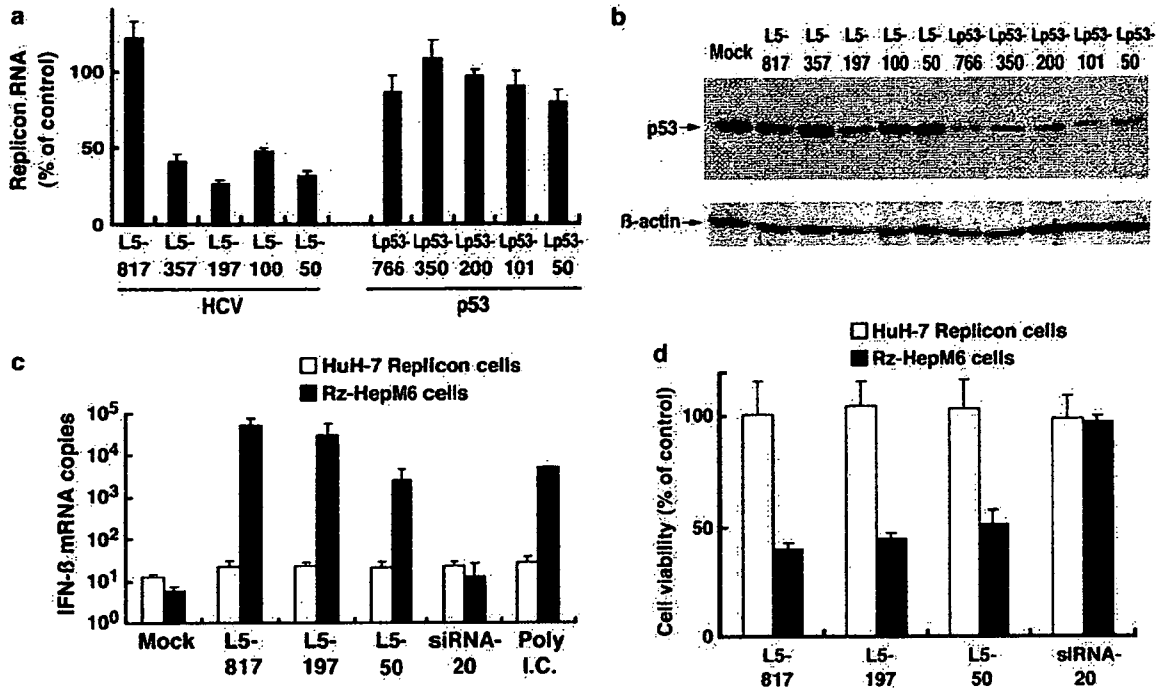

Figure 4 Transfection of long dsRNAs into HuH-7 replicon cells, which lack dsRNA-triggering IFN response, induces target-specific silencing. (a) R6CE2-N cells were transfected with long dsRNAs. Replicon RNA levels in cells transfected with 262 ng of dsRNA per 48-well dish were measured after 48 h by RTD-PCR. Data represent means ± s.d. ($n = 3$) of replicon levels compared with mock-transfected cells. (b) Immunoblot analysis of p53 and β-actin from replicon cells transfected with dsRNA targeting sites in the HCV genome or p53 mRNA. (c) Levels of human IFN-β mRNA were quantified by RTD-PCR 7 h after transfection with 50 ng of dsRNAs per 48-well dish. Values represent the mean copy number for each RNA per μg total RNA ± s.d. ($n = 5$). (d) Cell viability was determined after 48 h by WST-8 assay. Data represent means ± s.d. ($n = 3$) of WST conversion compared with mock-transfected cells.

death by apoptosis.[27] To examine the type I IFN response caused by direct transfection of dsRNA, we measured the intracellular IFN-β mRNA copy number and assessed cell viability. The IFN-β mRNA levels of R6FLR-N replicon cells (HuH-7 replicon cells) and the numbers of viable cells did not change following transfection with long dsRNAs or with the RNA duplex poly(rI):poly(rC) (Figures 4c and d). These results show that the dsRNA did not induce intracellular IFN-β mRNA or enhance apoptosis in HuH-7 replicon cells.

HuH-7 replicon cell lines are used as models for HCV replication and do not respond to the IFN signals.[28] We therefore investigated the effect of dsRNAs on the IFN-β response in another cell type. As an alternative model, we used HepG2 cells stably expressing the full genome HCV RNA (Rz-HepM6 cells).[29] Transfection with poly (rI):poly(rC) or long dsRNAs induced an IFN-β mRNA level of 10^3–10^5 copies per μg total RNA, whereas siRNA-20, a 20-nt duplex, induced only 10 copies per μg total RNA (Figure 4c). Furthermore, the number of viable Rz-HepM6 cells was reduced by transfection with long dsRNAs, but not with siRNA-20 (Figure 4d). These results indicated that direct transfection with dsRNA duplex longer than 50 nt induces IFN-β mRNA and causes cytotoxicity in Rz-HepM6 cells, but not in HuH-7 replicon cells. Therefore, to observe the knockdown efficiency of long-dsRNA against the HCV replicating model and the IFN response induced by long dsRNA, we tested the effects of RNAi in HuH-7 replicon, Rz-HepM6 and HepG2 cells.

U6 promoter-driven expression of long hairpin-RNA with mutations in the sense strand causes gene silencing without triggering an interferon response or cell death

We examined the ability of a stable hairpin-type siRNA-expression vector[30–32] to silence the HCV genome. Recently, U6 promoter-driven transcription of hRNA with mutations in the sense strand has been reported to be more effective for RNAi than hRNA containing nonmutated sense strands.[32] Therefore, we constructed vectors for U6 promoter-driven expression of hRNAs containing multiple mutations (mhRNA) and examined their ability to cause gene silencing. To confirm the RNAi effect, we transfected the long mhRNA-expression vectors into R6FLR-N replicon cells. The 50- and 197-bp mhRNA vectors against the HCV sequence reduced luciferase activity as effectively as the siE-20-bp mhRNA vector (Figure 5a). Furthermore, in Rz-HepM6 cells, the 50- and 197-bp mhRNA vectors targeted to the HCV sequence specifically suppressed HCV core protein expression (Figure 5b). To avoid the inhibition of IFN-β activation by HCV itself,[28] we next examined the IFN response in original HepG2 cells. In contrast to the direct transfection of dsRNAs targeted to the same sequences, the 50- and 197-bp mhRNA vectors did not induce the expression of IFN-β mRNA (Figure 5c). Owing to palindrome structure-specific recombination in the mammalian gene,[33] it was not possible to construct stably transformed cells expressing hRNA vectors against the

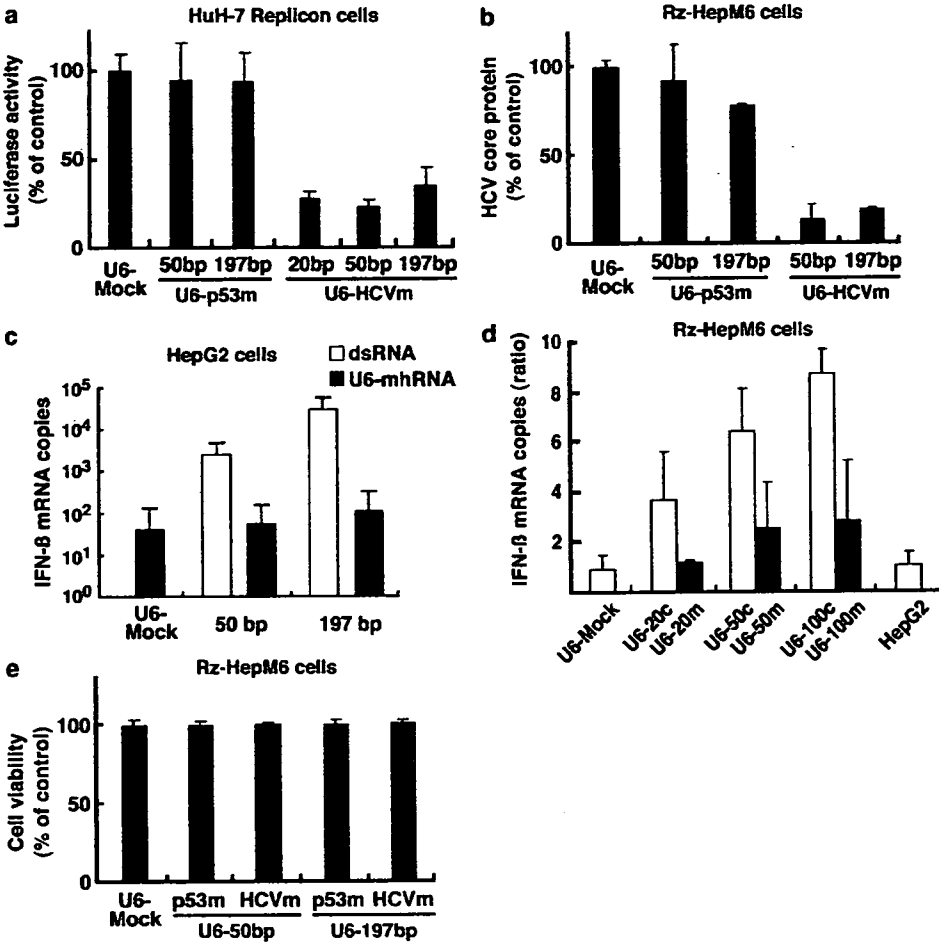

Figure 5 U6 promoter-driven expression of long mhRNA causes gene silencing without triggering an IFN response or cell death. (a) U6 promoter-driven transcription of long hRNAs containing multiple point mutations within the sense strand induced RNAi in R6FLR-N replicon cells. Luciferase activity was measured 96 h after transfection with 200 ng vector per 96-well dish. Data represent means ± s.d. compared with control U6 vector-transfected cells ($n = 3$). (b) Gene silencing for the long mhRNA-expression vector in Rz-HepM6 cells. All assays were performed 120 h after transfection with 600 ng vector per 48-well dish. Data represent means ± s.d. compared with control U6 vector-transfected cells ($n = 3$). (c) Interferon responses in original HepG2 cells following targeting of the same HCV sequences by direct transfection with dsRNA and U6 promoter-driven expression of mhRNA. The level of human IFN-β mRNA was measured by RTD-PCR 7 and 16 h after transfection. Values represent the mean copy numbers for each RNA per μg total RNA ± s.d. ($n = 3$). (d) U6 promoter-driven expression of mhRNA caused a lower IFN response in Rz-HepM6 cells than expression of hRNA containing non-mutated sense strands. The level of human IFN-β mRNA was measured by RTD-PCR 16 h after transfection. Values represent the mean ratios compared to control U6 vector-transfected cells ± s.d. ($n = 3$). U6-20c, HCVc-20bp; U6-20m, HCVm-20bp; U6-50c, U6-50m, U6-100c, and U6-100m are U6 vectors against the luciferase gene. (e) Cell viability of Rz-HepM6 cells was determined after 120 h by WST-8 assay. Data represent means ± s.d. ($n = 3$) of WST-8 conversion compared with control U6 vector-transfected cells.

HCV sequence containing non-mutated sense strands longer than 50 bp. Using control vectors against the luciferase gene (U6-50c, U6-50m, U6-100c and U6-100m), we confirmed an intracellular IFN response. In Rz-HepM6 cells, all of the mhRNA vectors (HCVm-20 bp, U6-50m and U6-100m) had a reduced IFN response compared to the hRNA vectors containing non-mutated sense strands (HCVc-20 bp, U6-50c and U6-100c; Figure 5d). Moreover, U6 promoter-driven expression of long mhRNAs against the HCV sequence was not cytotoxic (Figure 5e).

These results indicated that in IFN-responsive cells, U6 promoter-driven expression of modified long dsRNA, which be made by inserting multiple mutations in the sense strand of hRNA, can effectively and specifically silence HCV without triggering the IFN response or cell death.

Discussion

Previous studies have shown that HCV RNA can be suppressed by the RNAi machinery in replicon cells.[13–18] We demonstrated that there are two significant limitations for the use of siRNA-mediated RNAi as a therapy for HCV: first, it is difficult to predict which target site will be most effective for siRNA; and, second, it is difficult to target the other HCV genotypes with multiple sequences. We further examined the ability of d-siRNAs and intracellular-diced long dsRNAs to overcome these problems and inhibit HCV replication in HCV replicon

cells. We found that *ex-vivo* dicer-generated siRNAs generated from the 5′-UTR sequences are more effective for silencing than the most potent synthetic siRNA, siE. Our results further demonstrated that 50- and 197-bp dsRNA regions of the HCV genome are potential target areas for RNAi. Although dsRNA duplexes targeting the 50- to 357-bp sites in the HCV genome efficiently cause target silencing, dsRNA duplexes targeting the 817-bp HCV genome are less effective for HCV replication. This suggests that the area of the HCV genome that can be targeted by the RNAi machinery is restricted because of the formation of a complex internal ribosome entry site structure. Recently, Kim *et al.*[23] showed that 27-mer duplexes that are substrates of cellular dicer have enhanced RNAi potency and efficacy in mammalian cells. Our results also suggest that siRNAs generated by dicer from dsRNA duplexes longer than 50 nt are available in their natural form and, therefore, can have enhanced efficacy for RNAi.

In HuH-7 HCV replicon cells, which lack a long dsRNA-induced IFN response, the long dsRNAs were effective at causing RNAi of the HCV genome or endogenous p53. Therefore, we further examined the effect of dsRNA on HepG2 cells, in which dsRNA causes production of IFN-β and activates downstream signaling, including 2′–5′-oligoadenylate synthetase and protein kinase R.[29] Although transfection with dsRNA duplexes longer than 50 nt induced IFN-β and caused cell death, U6 promoter-driven expression of long hRNAs containing multiple point mutations in the sense strand (i.e., near-complementary inverted repeats) efficiently inhibited HCV replication, but was not cytotoxic. Moreover, the intracellular IFN-β mRNA titer was equivalent to that induced by the control U6 vector. The precise mechanism is now under investigation, but it is clear that this system allows intracellular-diced long dsRNA to induce RNAi without activating the IFN response in mammalian cells.

The genotype 2a-derived d-siRNAs generated from the 197-bp dsRNA were able to efficiently inhibit HCV genotype 1b replication. Thus, siRNAs generated from long dsRNA can cause silencing of heterogeneous viruses and should be able to overcome siRNA escape mutations. Long-term HIV-1 replication assays[34] revealed that, after 3–6 weeks of culture, siRNA-mediated RNAi-resistant viruses containing nucleotide substitutions or deletions in the target sequence arise. Wilson *et al.*[35] reported that HCV replicons escaped RNAi induced by subsequent treatment with the same siRNA directed against the NS5B cording region. In contrast, we also examined the long-term efficiency of long dsRNA-mediated RNAi using HCV replicon cells. When examined over 5 weeks with continuous transfection of 197-bp dsRNA, the HCV replicon RNA titer gradually decreased to a 100-fold reduction and never rebounded (data not shown). The degree of sequence conservation reflects the fact that the structural elements in the 5′- and 3′-terminal regions of the RNA are essential for viral replication.[36,37] Therefore, long dsRNA-mediated RNAi targeting a site in the 5′-UTR can avoid the problem of escape virus generation because extensive alterations in a conserved region of the viral genome would be required.

In summary, our results show that dicer-generated siRNAs from long dsRNA are highly effective for RNAi of the HCV genome and overcome genotype variations. We also showed that U6 promoter-driven expression of modified long dsRNA avoids activation of the IFN response and the induction of cell death normally caused by dsRNA. This strategy should be useful for therapy against natural viral infection by HCV and other RNA viruses, such as HIV-1, that display a high rate of mutation.

Materials and methods

Small interfering RNAs

We synthesized T7 siRNAs using the Silencer siRNA Construction Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The sense sequences of siRNAs were as follows:

siA (nt 26–45), 5′-ACUCCACCAUAGAUCACUCCUU-3′;
siB (nt 53–73), 5′-GGAACUACUGUCUUCACGCAGUU-3′;
siC (nt 139–159), 5′-GCCAUAGUGGUCUGCGGAACC UU-3′;
siD (nt 278–299), 5′-AGGCCUUGUGGUACUGCCUGAU UU-3′;
siE (nt 325–344), 5′-GUCUCGUAGACCGUGCAUCAUU-3′;
siF (nt 368–387), 5′-AGAAAAACCAAACGUAACACUU-3′;
siG (nt 9517–9537), 5′-GGCUCCAUCUUAGCCCUAGU CUU-3′;
siH (nt 9540–9560), 5′-GGCUAGCUGUGAAAGGUCCG UUU-3′; and
siI (nt 9553–9572) and 5′-AGGUCCGUGAGCCGCAUGA CUU-3′.

The sense sequence of the p53 m siRNA, which contains two nucleotide mismatches in the target sequence,[38] was 5′-GACUCCAGUGAUAAUCUGCUU-3′ (nucleotide mismatches underlined).

Long double-stranded RNAs

Long dsRNAs were prepared by *in vitro* transcription of PCR-amplified DNA templates. A modified T7 promoter sequence was added to the 5′-end of each PCR primer for amplification (Table 1). The dsRNAs were produced from the purified DNA templates using an Ampliscribe T7 transcription kit (Epicenter Technologies, Madison, WI, USA). Single-stranded RNA was converted to dsRNA by allowing annealing the two strands. Purification of dsRNA was performed as described for dicer-generated siRNAs.

Dicer-generated small interfering RNAs

Digestion with rhDicer (Gene Therapy Systems, San Diego, CA, USA) was carried out according to the manufacturer's protocol. The rhDicer-cleaved siRNAs and dsRNAs were separated by electrophoresis on a nondenaturing 12% polyacrylamide gel and detected by ultraviolet shadowing on a Fluor-coated thin-layer chromatography plate (Ambion). The rhDicer-cleaved siRNAs migrating as 20- to 21-bp bands were excised from the gel and extracted at 37°C for 4 h in extraction buffer (0.5 M ammonium acetate, 1 mM EDTA and 0.2% SDS). Following buffer exchange and desalting by gel filtration with Sephadex G-25 (Amersham Biosciences, Piscataway, NJ, USA), the rhDicer-cleaved siRNAs were dissolved in TE buffer. The cleaved siRNAs were then quantified by adsorption at 260 nm and stored at −70°C.

Table 1 Templates, PCR primers and amplicons used for the generation of dsRNAs

Template (source[a])	*Primer set*	*Primer sequence (5′ to 3′)*	*Amplicon name*	*Start position*	*Stop position*	*dsRNA size (nt)*
HCR6 genotype 1b (AY045702[a])	F-1	GCG TAA TAC GAC TCA CTA TAG GGA GAG AGT GCC CCG GGA GGT CTC GTA GAC	L5-50	311	360	50
	R-1	GCG TAA TAC GAC TCA CTA TAG GGA GAT TAG GAT TTG TGC TCA TGA TGC ACG				
	F-2	GCG TAA TAC GAC TCA CTA TAG GGA GAT AGT GTT GGG TCG CGA AAG GCC TTG	L5-100	261	360	100
	R-1	GCG TAA TAC GAC TCA CTA TAG GGA GAT TAG GAT TTG TGC TCA TGA TGC ACG				
	F-3	GCG TAA TAC GAC TCA CTA TAG GGA GAT GGA TCA ACC CGC TCA ATG CCT GGA	L5-197	201	397	197
	R-2	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG CGG TTG GTG TTA CGT TTG G				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-357	41	397	357
	R-2	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG CGG TTG GTG TTA CGT TTG G				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-572	41	612	572
	R-3	GCG TAA TAC GAC TCA CTA TAG GGA GAC CCT CGT TGC CAT AGA GGG GCC A				
	F-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA CTC CCC TGT GAG GAA CTA CTG TCT	L5-817	41	857	817
	R-4	GCG TAA TAC GAC TCA CTA TAG GGA GAA ACC GGG CAA ATT CCC TGT TGC ATA				
	F-5	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGG GGG AGA GAT ATA TCA CAG C	L3-345	9267	9611	345
	R-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA CAT GAT CTG CAG AGA GGC CAG T				
	F-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA GGA TGA TTC TGA TGA CCC ATT TCT	L3-674	8864	9537	674
	R-7	GCG TAA TAC GAC TCA CTA TAG GGA GAG ACT AGG GCT AAG ATG GAG CCA CCA				
	F-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA GGA TGA TTC TGA TGA CCC ATT TCT	L3-748	8864	9611	748
	R-6	GCG TAA TAC GAC TCA CTA TAG GGA GAA CAT GAT CTG CAG AGA GGC CAG T				
HCR24 genotype 2a (AY746460[a])	F-2a	GCG TAA TAC GAC TCA CTA TAG GGA GAT GGA TAA ACC CGC TCT ATG CCC GGC	2a-197	198	394	197
	R-2a	GCG TAA TAC GAC TCA CTA TAG GGA GAG CGA CGG TTA GTG TTT CTT TGG G				
p53 (NM_000546[a])	F-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC ATC ACA CTG GAA GAC TCC AG	Lp53-50	1013	1062	50
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p2	GCG TAA TAC GAC TCA CTA TAG GGA GAG TGT AAC AGT TCC TGC ATG GG	Lp53-101	962	1062	101
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p3	GCG TAA TAC GAC TCA CTA TAG GGA GAG TAT TTG GAT GAC AGA AAC ACT TTT CGA C	Lp53-200	863	1062	200
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p4	GCG TAA TAC GAC TCA CTA TAG GGA GAC ACC CGC GTC CGC GCC ATG G	Lp53-350	713	1062	350
	R-p1	GCG TAA TAC GAC TCA CTA TAG GGA GAC AAA GCT GTT CCG TCC CAG TAG				
	F-p5	GCG TAA TAC GAC TCA CTA TAG GGA GAG CAA TGG ATG ATT TGA TGC TG	Lp53-766	366	1131	766
	R-p2	GCG TAA TAC GAC TCA CTA TAG GGA GAC CCC TTT CTT GCG GAG ATT C				

dsRNA = double-stranded RNA; PCR = polymerase chain reaction.
[a]GenBank accession number.

Construction of U6 vectors

Plasmids containing a human U6 promoter were prepared as described previously.[30] A series of long-hairpin–RNA expression vectors was constructed by inserting a sense sequence between the U6 promoter and the corresponding antisense sequence. Sequences downstream of the U6 promoter were as follows (nucleotide substitutions underlined and loop sequence indicated in lowercase letters):

HCVc-20 bp, 5′-GTCTCGTAGACCGTGCATCAtagaatt acatcaagggagatTGATGCACGGTCTACGAGACTTTTT-3′;

HCVm-20 bp, 5′-GTCTTGTAGATTGTGTATTAtagaatt acatcaagggagatTGATGCACGGTCTACGAGACTTTTT-3′;

p53m-50 bp, 5′-CATTACATTGGAGGATTCCAGTGGT GATCTATTGGGGCGGAGTAGCTTTGgtgtgctgtccCA AAGCTGTTCCGTCCCAGTAGATTACCACTGGAGT CTTCCAGTGTGATGTTTTT-3′;

HCVm-50 bp, 5′-GAGTGTTCTGGGAGGTTTCGTAG ATCGTGTATCGTGAGTACAAGTTCTAAgtgtgctgtccT TAGGATTTGTGCTCATGATGCACGGTCTACGAGA CCTCCCGGGGCACTCTTTTT-3′;

p53m-197 bp, 5′-GTGTTTGGGTGATAGAGACACCTC TCGGCATGGTGTGGTGGTGTCTTATGAGTCGCT TGGGGTTGGTTCTGATTGTATCACTATCTATTACA GCTACGTGTGTGATAGTTCTTGTATGGGTGGCATG GACCGGGGGTCCATTCTCATCATTATCGCACTGG GAGATTCTAGTGGTGATCTATTGGGGCGGGACGG CTTTGgtgtgctgtccCAAAGCTGTTCCGTCCCAGTAG ATTACCACTGGAGTCTTCCAGTGTGATGATGGTG AGGATGGGCCTCCGGTTCATGCCGCCCATGCAG GAACTGTTACACATGTAGTTGTAGTGGATGGTGG TACAGTCAGAGCCAACCTCAGGCGGCTCATAGG GCACCACCACACTATGTCGAGAAGTGTTTCTGTC ATCCAAATACTTTTT-3′;

HCVm-197 bp, 5′-ATGGGTCAGCTCGTTCAATGCTT GGAGGTTTGGGTGTGTCCTCGTGAGATTGCTAGT CGAGTGGTGTTGGGTTGCGGAAGGTCTTGTGGTG CTGTCTGATGGGGTGTTTGTGAGTGTCCTGGGAG GTTTCGTTGACTGTGCATTATGAGTACAGATCCTA GACCTCAGAGAAGGACCAGACGTGACATCAACT GCCGCgtgtgctgtccGCGGCGGTTGGTGTTACGTTTG GTTCTTCTTTGGGGTTTAGGATTTGTGCTCATGAT GCACGGTCTACGAGACCTCCCGGGGCACTCGCA AGCACCCTATCAGGCAGTACCACAAGGCCTTTC GCGACCCAACACTACTCGGCTAGCAGTCTCGCG GGGGCACGCCCAAATCTCCAGGCATTGAGCGGG TTGATCCATTTTT-3′;

U6-50c, 5′-GCCTTCAGGATTACAAGATTCAAAGTG CGCTGCTGGTGCCAACCCTATCTttcaagagaGAATA GGGTTGGCACCAGCAGCGCACTTTGAATCTTGTA ATCCTGAAGGCTTTTT-3′;

U6-50m, 5′- GCCTTTAGGATTATAAGGTTCAAAGTG TGCTGTTGGTGTCAACTCTATCTttcaagagaGAATAG GGTTGGCACCAGCAGCGCACTTTGAATCTTGTAA TCCTGAAGGCTTTTT -3′;

U6-100c, 5′-GATTTCGAGTCGTCTTAATGTATAGATT TGAAGAAGAGCTGTTTCTGAGGAGCCTTCAGGA TTACAAGATTCAAAGTGCGCTGCTGGTGCCAACC CTATTCttcaagagaGAATAGGGTTGGCACCAGCAGC GCACTTTGAATCTTGTAATCCTGAAGGCTCCTCA GAAACAGCTCTTCTTCAAATCTATACATTAAGAC GACTCGAAATCTTTTT-3′ and

U6-100m, 5′-GATTTCGGGTTGTCTTGATGTATGGGT TTGGAGAGGAGTTGTTTCTGGGGAGTCTTTAGGA TTATAAGGTTCAAAGTGTGCTGTTGGTGTCAACT CTATTCttcaagagaGAATAGGGTTGGCACCAGCAGC GCACTTTGAATCTTGTAATCCTGAAGGCTCCTCA GAAACAGCTCTTCTTCAAATCTATACATTAAGAC GACTCGAAATCTTTTT-3′.

Construction of recombinant plasmids for expressing the hepatitis C virus replicon

The HCV genotype 1b replicon pRep-R6FLR-NRz was assembled and cloned from pRep-R6Rz and the 1bneo/delS plasmid.[39] Replicon pRep-R6Rz was engineered from pHCR6-Rz[29] as described previously,[20] and replicon pRep-R6-NRz was engineered by replacing a NS3-NS5B fragment (nt 3420–7996; *Mfe*I site) in pRep-R6Rz with a NS3-NS5B fragment (nt 3420–7996; *Mfe*I site) from the 1bneo/delS plasmid. The final replicon, pRep-R6FLR-NRz, was constructed by replacing the neomycin phosphotransferase (*neo*^{r}) gene of pRep-R6-NRz with a chimeric gene encoding firefly luciferase protein fused in-frame with the 2A genes of FMDV and *neo*^{r}.

The HCV genotype 1b replicon pRep-R6CE2-NRz was assembled and cloned from pRep-R6-NRz and pHCR6-Rz. Plasmid pRep-R6CE2-NRz was engineered by replacing the HCV internal ribosome entry site gene (nt 1–389) in pRep-R6-NRz with a Core-NS2 gene (nt 1–3030; *Rsr*II site) from the pHCR6 plasmid. The pRep-R6CE2-NRz replicon was constructed by fusing the HCV NS2 protein gene in-frame with the genes for FMDV 2A protein and *neo*^{r}.

Cell culture and transfection

We maintained the human hepatoma cell line HuH-7 in complete Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA). G418 was added to a final concentration of 500 μg/ml to cell lines carrying HCV replicons.[20] Replicon cells were transfected with synthetic siRNA using Trans IT TKO reagent (Mirus, Madison, WI, USA) or with modified siE, dicer-generated siRNAs, long dsRNA and DNA vector using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Also, Rz-HepM6 cells[29] were transfected with various amounts of dsRNAs or DNA vector using Lipofectamine 2000.

Luciferase assays

The luciferase assay was performed using the Steady-Glo or Bright-Glo luciferase assay systems (Promega). Luciferase activities were quantified using a luminometer (Mithras LB940; Berthold Technologies, Wildbad, Germany).

Cell viability assay

To evaluate the cytotoxic effects of dsRNAs, cell viability was measured by metabolic conversion of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) using a Cell Counting Kit-8 (Wako, Tokyo, Japan) according to the manufacturer's protocol.

Immunoblot analysis

Immunoblot analysis was performed as described previously.[29] Anti-p53 (Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) and anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as the primary antibodies.

Real-time detection–polymerase chain reaction analysis

The HCV genome RNA and IFN-β mRNA were quantified using the ABI PRISM 7700 sequence detector (Applied Biosystems, Foster City, CA, USA) as described previously.[24,29]

Quantification of hepatitis C virus core protein

Hepatitis C virus core protein was assessed in cell lysates using a fluorescent enzyme-linked immunosorbent assay.[40]

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Liver target delivery of small interfering RNA to the HCV gene by lactosylated cationic liposome☆

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***Background/Aims*: RNA interference has considerable therapeutic potential, particularly for anti-viral therapy. We previously reported that hepatitis C virus (HCV)-directed small interfering RNA (siRNA; siE) efficiently inhibits HCV replication, using HCV replicon cells. To employ the siRNA as a therapeutic strategy, we attempted *in vivo* silencing of intrahepatic HCV gene expression by siE using a novel cationic liposome.**

***Methods*: The liposomes consisted of conjugated lactose residues, based on the speculation that lactose residues would effectively deliver siRNA to the liver *via* a liver specific receptor. The lactosylated cationic liposome 5 (CL-LA5) that contained the most lactose residues introduced the most siRNA into a human hepatoma cell line, which then inhibited replication of HCV replicons.**

***Results*: In mice, the siRNA/CL-LA5 complexes accumulated primarily in the liver and were widespread throughout the hepatic parenchymal cells. Moreover, siE/CL-LA5 specifically and dose-dependently suppressed intrahepatic HCV expression in transgenic mice without an interferon response.**

***Conclusions*: The present results indicate that the CL-LA5 we developed is a good vehicle to lead siRNA to the liver. Hence, CL-LA5 will be helpful for siRNA therapy targeting liver diseases, especially hepatitis C.**

***Keywords*: siRNA; HCV; Cationic liposome; Lactosylation; Target delivery; Hepatocyte; Liver; Transgenic mouse; Interferon**

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Abbreviations: HCV, hepatitis C virus; RNAi, RNA interference; siRNA, small interfering RNA; IFN, interferon; UTR, untranslated region; CL-LA, lactosylated cationic liposome; siE, HCV-directed siRNA.

1. Introduction

Hepatitis C virus (HCV) is a major etiological agent that causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. Although combination therapy with pegylated interferon-α and ribavirin has markedly improved the clinical outcome, less than half of the patients with chronic hepatitis C can be expected to respond favourably to currently available agents [2]. Therefore, developing a specific reagent against HCV is a major public health objective.

RNA interference (RNAi) is one type of post-transcriptional gene silencing [3,4]. The effector of RNAi is

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short interfering RNA (siRNA) duplexes (~21–23 nt), which play a key role in the specific degradation of target mRNA. Currently, RNAi technology is widely used as a tool for gene function analysis. In addition, it is expected to be a powerful therapeutic agent to silence pathogenic gene products associated with disease, including cancer, viral infections and autoimmune disorders [5–10]. Previously, we and others reported that synthetic siRNA efficiently and specifically inhibits HCV replication *in vitro* [11–20] and suggested the potential for siRNA as a novel HCV agent.

In fact, the RNAi machinery has been shown to work *in vivo* by injection of siRNA [21]. However, safety and delivery remain the main obstacles to achieving *in vivo* gene silencing by RNAi technology. Currently, viral vectors [22], hydrodynamic injection [23] and cationic liposomes [24] have been the main methods of introducing siRNA *in vivo*. However, the mechanism of action of viral vectors has not been clarified and may result in severe side effects. Furthermore, hydrodynamic injection cannot be used for human therapy. On the other hand, since the physical properties of cationic liposomes are well understood, the use of these liposomes holds the best promise for clinical application. In addition, cationic liposomes do not elicit an immune response, which is a great advantage for drug targeting in that multiple administrations of siRNA are possible, which is crucial for an siRNA therapeutic effect. Moreover, cationic liposomes are easily modified and improved.

In HCV therapy, it is important that reagents are specifically led to the liver. Thus, to specifically and effectively transfer siRNA into hepatocytes, we designed lactosylated cationic liposomes, as Ohishi et al. reported that lactosylated polyion complex micelles enhanced the delivery of oligonucleotides into hepatoma cells [25]. Based on their observations we expected that siRNA complexed with lactosylated cationic liposomes would be superficially trapped in the liver by lactose-specific receptors and therefore effectively introduced into hepatic parenchymal cells *in vivo*.

Here, we report that siRNAs with cationic liposomes containing lactose residues were largely transfected into hepatocytes *in vitro* and *in vivo*, where they efficiently suppressed intrahepatic HCV expression in transgenic mice. Furthermore, this system did not activate the interferon (IFN) system. Our results strongly suggest that lactosylated cationic liposomes have an appropriate mechanism by which to deliver siRNA as a therapy for liver disease.

2. Materials and methods

2.1. siRNAs

The design of HCV-directed siRNA has been described previously [11]. Briefly, we designed nine siRNAs that target the 5′-UTR and 3′-UTR of the HCV genome and examined the efficiency of their inhibition of HCV replication *in vitro*. Among the nine siRNAs, the most effective siE was used in the present study and was directed toward nucleotides 325–344 of the HCV genome. The target sequence was 5′-GUCUC GUAGACCGUGCAUCAUU-3′. The p53m siRNA (sip53m) [11] and GL3-M1 siRNA (siGL3-M1) were used as the negative controls. The sense sequence of siGL3-M1, which is sequence-specific for firefly luciferase mRNA, was 5′-GCUAUGAAACGAUAUGGGCUU-3′.

2.2. Preparation of cationic liposomes and siRNA/cationic liposome complexes

The cationic liposomes were composed of three lipids: a cationic lipid, phosphatidylcholine (PC), and lactosylated phosphatidylethanolamine (LA-PE). The preparation of the cationic liposomes [24] and the synthesis of the LA-PE [26] have been described previously. The ratio of the two neutral lipids, PC and LA-PE in the liposomes was as follows: CL-LA0, 5:0; CL-LA1, 4:1; CL-LA2, 3:2; CL-LA3, 2:3; CL-LA4, 1:4; and CL-LA5, 0:5. Each siRNA was mixed with 16 times the amount of cationic liposome, resulting in siRNA/CL-LA. The size of every siRNA/CL-LA was controlled as an average 150 nm.

2.3. Inhibition assay of HCV replication in replicon cells

We used two kinds of HCV replicon cells [27]; FLR3-1 (genotype 1b, Con-1; Fig. 1a) [28] and R6FLR-N (genotype 1b, strain N) [11]. siRNA/CL-LA was added to the medium of the HCV replicon cells, FLR3-1 or R6FLR-N, at a final concentration of 30 nM. For positive control [11], HCV replicon cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After 72 h incubation, we performed luciferase assays using the Bright-Glo luciferase assay system (Promega, Madison, WI).

2.4. Immunoblotting

Cells were harvested using lysis buffer [11]. Then 5 μg of protein was separated by 10% SDS–PAGE, and electro-blotted onto a PVDF membrane (Immobilon-P; Millipore, Billerica, MA). Rabbit polyclonal anti-HCV nonstructural protein 3 (NS3) antibody (R212) prepared in our laboratory and mouse monoclonal anti-β-actin antibody (Sigma, St. Louis, MO) were used as the primary antibodies.

2.5. Transfection efficiency of siRNA by CL-LA in vitro

The transfection efficiencies of Cy3-labeled siRNA (Cy3-siRNA) by CL-LA were determined using confocal laser microscopy (Zeiss, Jena, Germany). HCV replicon cells were seeded in the Lab-Tek II Chamber Slide-System (Nalge Nunc International, Rochester, NY) at 2.0×10^4 cells per well. The siRNA was labeled with Cy3 using a Silencer siRNA Labeled Kit (Ambion, Austin, TX). After incubation for 24 h, the cells were fixed in 4% buffered formalin and the nuclei stained using DAPI.

2.6. Animals

Male BALB/c mice at 8 weeks of age were purchased from Japan SLC (Hamamatsu, Japan). BALB/c mice and CN2-29 transgenic mice received human care according to guidelines of the National Institutes of Health. Animal experiment protocols performed in accordance with The Tokyo Metropolitan Institute of Medical Science Animal Experiment Committee.

2.7. siRNA delivery by CL-LA in vivo

Alexa-546 or Alexa-568 labeled siE/CL-LA was intravenously injected into BALB/c mice. After 5 and 30 min, the liver, lung, spleen, and kidney were extirpated from the mouse. Sections of these tissues were then stained with DAPI and slides examined using confocal laser microscopy (Zeiss).

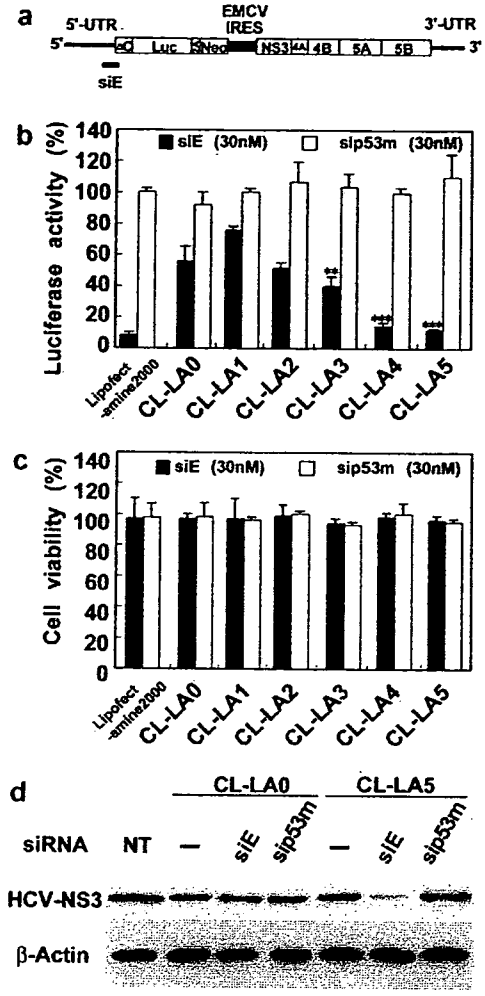

Fig. 1. Effect of siRNA/CL-LA in HCV replicon cells. (a) Schematic representation of HCV replicon RNA and siE position. UTR, untranslated region; ΔC, truncated HCV core region (nucleotides 342–377); Luc, firefly luciferase gene; 2A, 2A genes of foot-and-mouth disease virus; Neo, neomycin resistant gene; EMCV, encephalomyocarditis virus; IRES, internal ribosomal entry site; NS, HCV nonstructural protein; siE, HCV-directed siRNA. (b) FLR3-1 replicon cells were treated with 30 nM siRNA/CL-LA. Luciferase activity was measured after 72 h. Data represent means ± SD compared with mock-transfected cells ($n = 5$). The average luciferase activities were analyzed by Dennett's test. $^{**}P < 0.01$ vs. CL-LA0 and $^{***}P < 0.001$ vs. CL-LA0. sip53m was used as the negative control. Commercial transfection agent Lipofectamine 2000 was used as the relative positive control. (c) Cell viability was determined after 72 h. Data represent means ± SD ($n = 5$) of WST conversion compared with mock-transfected cells. (d) Immunoblot analysis of HCV-NS3 and β-actin. NT, non treatment.

2.8. Gene silencing of HCV genome expression in vivo by siRNA

We used 8- to 10-week-old, 20 g CN2-29 transgenic mice, which contain conditional HCV cDNA, the expression of which is regulated by the Cre/loxP-system (Fig. 4a) [29]. Expression of HCV core protein is regulated by Cre DNA recombinase in the liver, which can be expressed by administration of adenovirus encoded Cre DNA recombinase (AxCANCre). AxCANCre was intravenously administered at 2×10^9 pfu per body 1 h prior to siRNA (2.5, 5 or 10 mg/kg) injection. After 48 h, expression levels of HCV core protein in the liver were detected using the Ortho HCV core protein ELISA kit (Eiken Chemical, Tokyo, Japan).

2.9. Detection of interferon-β induction by administration of siRNA/CL-LA5 complex

Poly(I):poly(C) was purchased from Yamasa-shoyu (Chiba, Japan). siRNA/CL-LA5 or poly(I):poly(C)/CL-LA5 (200 μg) was intravenously injected into the CN2-29 mice. After 6 h, the livers were extirpated and total RNA was extracted by the acid guanidinium–phenol–chloroform method. cDNA was synthesized from 1 μg of the total RNA using TaqMan reverse transcription reagents (ABI, Foster City, CA). Expression levels of IFN-β mRNA were determined using a TaqMan gene expression assay kit (ABI) according to the manufacturer's instructions [30,31].

2.10. Statistical analysis

The data are expressed as means ± SD. Statistical analysis was conducted using the analysis of variance with the Dennett's test for multiple comparisons. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Optimization of amount of lactosylated phosphatidylethanolamine (LA-PE) included in the cationic liposomes

To optimize the amount of LA-PE in lactosylated cationic liposomes (CL-LA), we initially prepared six kinds of cationic liposomes containing various amounts of lactose residues, and investigated the inhibitory effects of siE/CL-LA against HCV replication in FLR3-1 replicon cells. The CL-LA strengthened the inhibitory efficiency of siE by increasing the amount of LA-PE. The siE/CL-LA5, which contained LA-PE but not PC, had the strongest inhibitory effect. On the other hand, none of the sip53m/CL-LA affected luciferase activity reflecting the HCV replication (Fig. 1b). To access cytotoxicity of a complex of siRNA and CL-LA, cell viability was measured by the WST-8 assay [11]. None of siE/CL-LA or sip53m/CL-LA showed any cytotoxicity (Fig. 1c). A luciferase assay in another replicon cell line, R6FLR-N replicon [11], showed similar results for HCV-specific silencing (data not shown). Immunoblot analysis showed that the levels of HCV NS3 protein that were translated from the HCV replicon were decreased by siE/CL-LA5, but not by sip53m/CL-LA5 (Fig. 1d). These results indicated that siE/CL-LA5 inhibited HCV replication the most effectively *in vitro* and that this inhibition was not due to nonspecific reduction caused by the complex of siRNA and CL-LA.

3.2. Transfection efficiency of siRNA by CL-LA5 into HCV replicon cells

To investigate whether lactose residue enhances the transfection of siRNA, we observed fluorescent-labeled siE introduced into RLR3-1 replicon cells. After a 24 h incubation with siE/CL-LA0 or siE/CL-LA5, the cells were observed by fluorescence microscopy (Fig. 2). The lactosylated cationic liposome CL-LA5 transfected siE into replicon cells more effectively than the non-lactosylated cationic liposome CL-LA0. Moreover, fluorescence of siE transfected by CL-LA5 was observed mainly in the cytoplasm, and was more effective than that with the commercial agent Lipofectamine 2000. These results demonstrated that the lactose residue very strongly enhanced the transfection efficiency of siRNA into replicon cells, particularly in the cytoplasm.

3.3. Delivery of siRNA by CL-LA5 in mice

Next, we investigated the delivery of siRNA by CL-LA5 in BALB/c mice, which were intravenously injected with fluorescent-labeled siE/CL-LA0 or siE/CL-LA5 (Fig. 3). At 5 and 30 min after injection *via* the orbital vein, the livers of the mice were extirpated and observed by fluorescence microscopy. The fluorescence intensity of siE/CL-LA5 at 5 min was clearly stronger than that of siE/CL-LA0. At 30 min after injection, fluorescence of siE/CL-LA5 was equally spread throughout the hepatic parenchymal cells, although that of siE/CL-LA0 could be patchily detected in parts (Fig. 3a). These results demonstrated that CL-LA5 could more easily take siRNA into the cytoplasm of parenchymal liver cells. Furthermore, we also examined the tissue distribution of siRNA delivered by CL-LA5 (Fig. 3b). At 30 min after injection, the liver, spleen, kidney and lung of another mouse were excised and the intensity of fluorescence of labeled-siE in these tissues calculated. Although the relative fluorescence of siE/CL-LA0 accumulated in the liver and spleen, that of siE/CL-LA5 accumulated primarily in the liver alone, and the residual fluorescence of siE was equally diffused in other tissues. Taken together, these results indicated that CL-LA5 was able to trap siRNA primarily in the mouse liver, where it could be efficiently taken up into the hepatocytes.

3.4. Down-regulation of HCV protein expression by siE/CL-LA5 in transgenic mouse liver

To extend our findings of the *in vitro* silencing effect by siE/CL-LA5 and *in vivo* siRNA delivery by CL-LA5, we performed an additional study in an HCV transgenic mouse model [29]. We administered siE/CL-LA5 to CN2-29 mice after inducing HCV protein expression by AxCANCre (Fig. 4b). The mice were sacrificed on the second day after injection, and expression of HCV core protein in the liver measured by ELISA. The siE/CL-LA5 decreased the amount of core protein in a dose-dependent manner. The maximal dose of HCV unrelated siGL3-M1/CL-LA5 did not inhibit the expression of HCV core protein. These results demonstrated that siE/CL-LA5 specifically inhibited HCV protein expression in mouse liver.

3.5. IFN response by siRNA/CL-LA5 in vivo

It has been reported that siRNA can activate the cellular interferon (IFN) pathway, especially when delivered by cationic liposome transfection reagents [32,33].

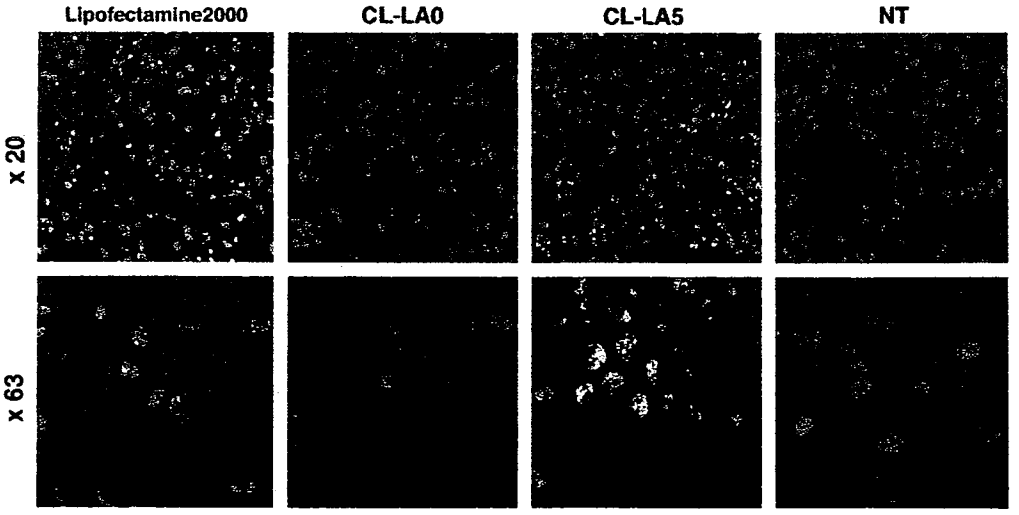

Fig. 2. Transfection efficiency of fluorescent-labeled siRNA into HCV replicon cells. FLR3-1 replicon cells were treated with CL-LA0, CL-LA5, or Lipofectamine 2000 complexed with Cy3-labeled siE (100 nM). After incubation for 48 h, the cells were observed by fluorescence microscopy. The nuclei were stained with DAPI. NT, non treatment.

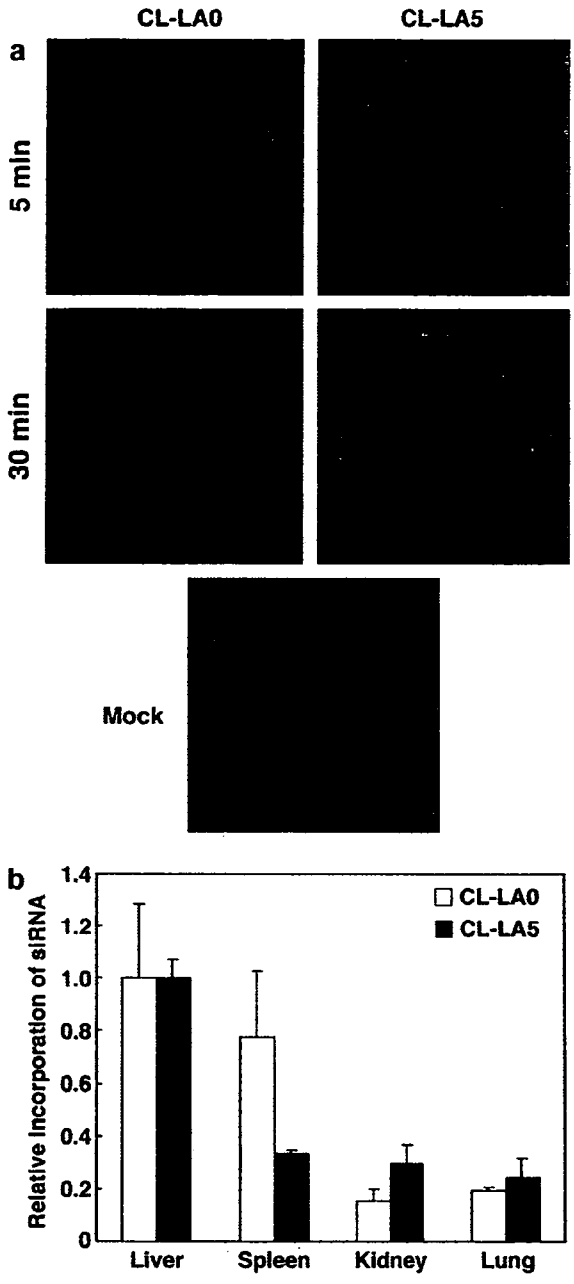

Fig. 3. siRNA delivery in mice. (a) Distribution characteristics of siRNA in liver. Alexa-546 labeled siE/CL-LA0 or siE/CL-LA5 (4.3 mg/kg) was injected intravenously into the orbital veins of BALB/c mice. The liver was observed by fluorescence microscopy at 5 or 30 min after injection. The nuclei were stained with DAPI. Mock; 10% (w/v) maltose solution. (b) Tissue distribution of Alexa-568 labeled siRNA delivered by CL-LA. The liver, spleen, kidney, and lung were examined at 30 min after injection, and the intensity of fluorescence of labeled-siE/CL-LA0 or siE/CL-LA5 was then calculated at 3 locations in each tissue specimen. The relative ratio for incorporation of siE was obtained by setting the liver intensity as control. Data represent means ± SD.

To examine whether a type I IFN response was caused by siRNA/CL-LA5, we measured IFN-β mRNA levels in the liver of CN2-29 mice. Poly(I):poly(C)/CL-LA5

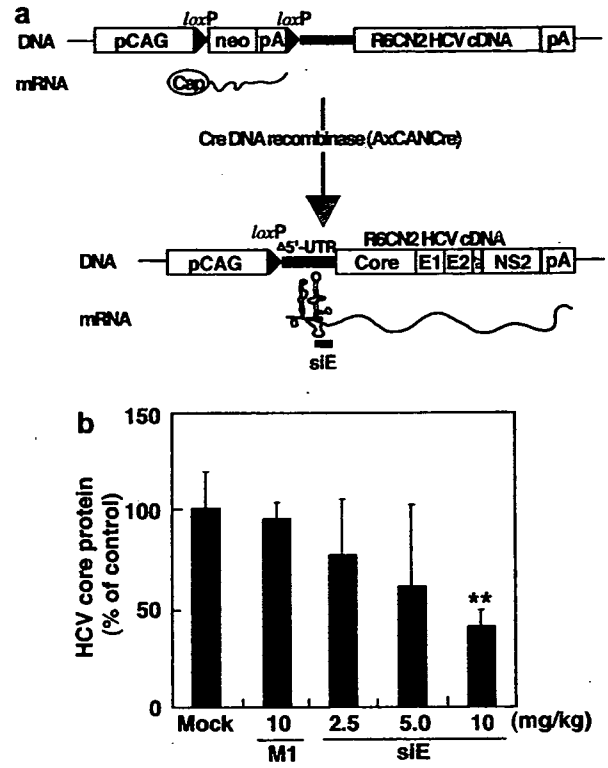

Fig. 4. Effect of siRNA on intrahepatic HCV expression in mice. (a) The CN2-29 transgenic mice contain part of the HCV gene (part of the 5′-UTR to NS2 protein). HCV protein expression can be controlled by infection of adenovirus encoding Cre DNA recombinase (AxCANCre). (b) siRNA/CL-LA5 was intravenously injected into CN2-29 mice at 1 h after AxCANCre infection. HCV core protein expressed in the liver was detected by ELISA after 48 h ($n = 3$). The average HCV core proteins were analyzed by Dennett's test. $^{**}P < 0.01$ vs. Mock control livers. Mock; 10% (w/v) maltose solution, M1; siGL3-M1.

drastically increased the IFN-β mRNA level to 10^6–10^7 copies per 1 μg total RNA, whereas siRNA/CL-LA5 induced only 10^1–10^2 copies per 1 μg total RNA

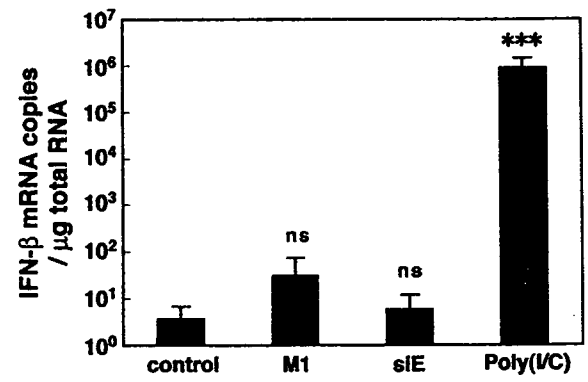

Fig. 5. IFN response *in vivo*. siRNA complexed with CL-LA5 was intravenously injected into CN2-29 mice ($n = 3$). At 6 h after injection, the IFN-β mRNA copy number was measured using real-time detection PCR. The average IFN-β mRNA copies were analyzed by Dennett's test. $^{***}P < 0.001$ and ns vs. non-treated control mice livers. ns, not significant; M1, siGL3-M1; Poly(I/C), poly(I):poly(C).

(Fig. 5). Moreover, siE/CL-LA5 induced only below 10^1 copies per 1 μg total RNA, equal to non-treated mouse liver. These results indicated that CL-LA5 was able to lead siRNA and poly(I):poly(C) to the liver by systemic intravenous injection. Subsequent administration of siE/CL-LA5 was then unable to activate the IFN response in mouse liver.

4. Discussion

Many studies of delivery systems for siRNA based on cationic liposomes have already been reported [24,34–40]. In those studies, the major problem of liposome as an siRNA carrier appears to have been a limitation to specific cell types, which resulted in unwanted tissue distribution *in vivo*. To address this problem, ligand or receptor mediated siRNA delivery systems were developed and these were able to increase uptake into the target cells [41–43]. In this study, to achieve liver specific delivery of siRNA, we designed a lactosylated cationic liposome as a carrier of siRNA and evaluated its delivery ability. The galactose terminus of lactose is a ligand of the asialoglycoprotein receptor, which is specifically expressed on the surface of hepatocytes. Thus, we expected liver specific delivery of siRNA would be enabled *via* this receptor-mediated endocytotic pathway [44]. As expected, CL-LA5, composed of cationic lipid and lactosylated phosphatidylethanolamine, effectively delivered the siE, which then inhibited HCV gene expression *in vitro* and *in vivo*.

siRNA is able to activate the cellular interferon pathway, especially when delivered with cationic liposome transfection reagents [32,33]. In addition, recent reports have revealed that siRNAs containing the 5′-UGUGU-3′ sequence are able to induce a toll-like receptor-mediated IFN response only when they are delivered *in vivo* with cationic lipid through intravenous administration [45,46]. These issues have raised concerns about the future of siRNA therapeutics. In fact, we found that the siE/CL-LA5 barely activated the type I IFN response, but that siGL3-M1/CL-LA5 weakly induced this response in mouse liver, although neither agent contained the 5′-UGUGU-3′sequence. Although the reasons for these phenomena are unclear, siE/CL-LA5 is likely to be tolerated by innate *in vivo* immunity and to have therapeutic potential for HCV.

On the other hand, we used transgenic mice expressing HCV RNA (encoding the IRES to NS2 protein region) to measure the knockdown efficiency of siE/CL-LA5. The target RNA is not replicable. During the course of an HCV infection, the virus exists as quasi-species composed of multiple variants [47]. Due to this physiological condition, mutants resistant to the siRNA may arise rapidly [48,49]. Although we believe that siE/CL-LA5 has the potential to silence natural HCV RNA, further investigations with an actual HCV infection system [50] are required.

Furthermore, siRNA/CL-LA5, a systemic method of delivery of siRNAs to liver tissue, would provide a means to introduce siRNAs into hepatocytes to achieve maximal therapeutic benefit, decrease the amount of drug required, and avoid nonspecific silencing and IFN response. Although further optimization of siRNA stability and safety profile characterizations are required for its practical application in humans, our delivery system of siRNA with CL-LA5 is a promising and feasible therapeutic strategy for liver disease associated with pathogenic gene products such as HCV.

Acknowledgements

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Host sphingolipid biosynthesis as a target for hepatitis C virus therapy

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An estimated 170 million individuals worldwide are infected with hepatitis C virus (HCV), a serious cause of chronic liver disease. Current interferon-based therapy for treating HCV infection has an unsatisfactory cure rate[1,2], and the development of more efficient drugs is needed. During the early stages of HCV infections, various host genes are differentially regulated[3], and it is possible that inhibition of host proteins affords a therapeutic strategy for treatment of HCV infection. Using an HCV subgenomic replicon cell culture system, here we have identified, from a secondary fungal metabolite, a lipophilic long-chain base compound, NA255 (1), a previously unknown small-molecule HCV replication inhibitor. NA255 prevents the *de novo* synthesis of sphingolipids, major lipid raft components, thereby inhibiting serine palmitoyltransferase, and it disrupts the association among HCV nonstructural (NS) viral proteins on the lipid rafts. Furthermore, we found that NS5B protein has a sphingolipid-binding motif in its molecular structure and that the domain was able to directly interact with sphingomyelin. Thus, NA255 is a new anti-HCV replication inhibitor that targets host lipid rafts, suggesting that inhibition of sphingolipid metabolism may provide a new therapeutic strategy for treatment of HCV infection.

The majority of acute hepatitis C virus (HCV) infections become chronic; some progress toward liver cirrhosis or hepatocellular carcinoma[4]. Currently, viral enzyme–targeted drugs are being developed on the basis of viral nonstructural (NS) proteins—NS3/4A serine protease and NS5B RNA-dependent RNA polymerase—and are currently under clinical investigation for the treatment of HCV infection[5,6]. However, resistance to antiviral agents directly targeting viral enzymes is a major factor limiting the efficacy of therapies against many retroviruses or RNA viruses owing to the error-prone nature of the viral reverse transcriptases or RNA-dependent RNA polymerases[7]. As these HCV-specific inhibitors enter clinical trials, resistance could become a major problem in patients treated with drugs targeting viral proteins. Currently, an HCV subgenomic replicon cell culture system is used as the cell-based model to study HCV replication and host-cell interactions[8]. It provides a useful tool for HCV drug development as well as clarification of the mechanisms of HCV RNA replication. The replicon cell line #Huh-7/3-1 constitutively expresses an HCV subgenomic replicon (genotype 1b, HCV-Con1) and enables the quantification of replication levels by measuring luciferase, making it suitable for high-throughput screening of HCV replication inhibitors. Here, using replicon cells, we identified a previously unknown small-molecule HCV replication inhibitor with the potential to be a clinical drug candidate for the treatment of HCV.

To identify a lead compound inhibiting HCV replication, we performed standard cell-based high-throughput screening using natural product libraries derived from microbial and fungal metabolites. We selected several hits that showed HCV replication inhibitory activity without host cellular toxicity. The most active extracts derived from *Fusarium* sp. led to the isolation of NA255 (**1**; **Fig. 1a**). The luciferase activities of the HCV replicon cells showed that replication was suppressed by NA255 in a dose-dependent manner with a mean of 50% inhibitory concentration (IC_{50}) of 2 nM (**Fig. 1b**). In addition, levels of the replicon RNA significantly decreased after treatment with NA255 according to northern blot analysis (**Fig. 1c**). NA255 had no effect on host cell viability, as measured by the WST-8 assay (**Fig. 1b**, IC_{50} > 50 μM), total cell counts using the Trypan Blue exclusion test, or host cell cycle progression from flow-cytometry analysis (**Supplementary Fig. 1** online). We detected HCV-NS3 protein, which includes the protease and helicase domains, in HCV replicon cells using immunostaining analysis. In the absence of NA255 treatment, the NS3 protein was mainly localized in the perinuclear region (**Fig. 1d**, upper panel). After treatment with NA255 for 96 h, NS3 protein disappeared substantially (**Fig. 1d**, lower panel). Western blot analysis showed that NA255 treatment resulted in reduced levels of viral proteins such as NS3, NS5A and NS5B over time (**Fig. 1e**). These results indicate that NA255 is a potent small-molecule inhibitor of HCV replication.

To clarify the mode of action of NA255, we first evaluated inhibitory activity against the viral enzymes that are essential for HCV replication. NA255 did not significantly inhibit NS3 serine protease, NS3 helicase or NS5B RNA polymerase *in vitro*. In addition, there were no changes after treatment with NA255 in the expression levels of interferon (IFN)-induced antiviral response genes such as

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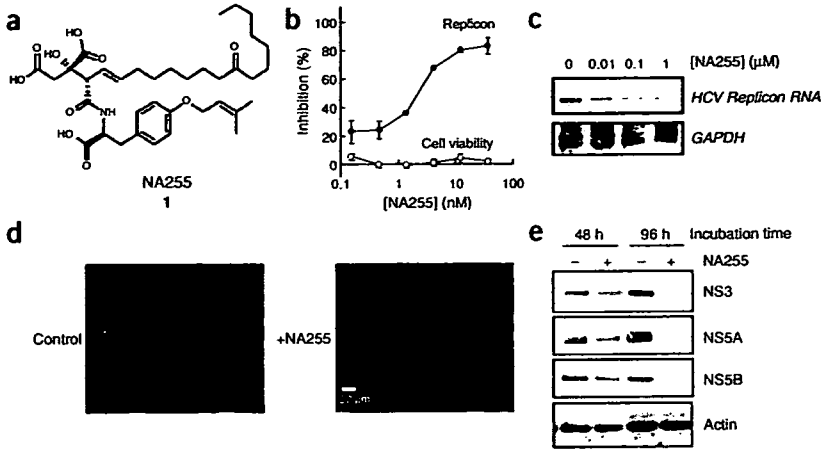

Figure 1 Identification of a small-molecule HCV replication inhibitor. (a) Chemical structure of NA255 (1). (b) HCV replicon cells were treated with various concentrations of NA255 for 72 h. Replication levels of *HCV* RNA were analyzed by luciferase assay, and cell viability was determined by WST-8 assay. The data represent the mean value, and the bars indicate the standard deviation of triplicate determination. (c) Total RNA was extracted from HCV replicon cells cultured in the presence of indicated concentrations of NA255 for 96 h and analyzed for the subgenomic *HCV* RNA and *GAPDH* RNA levels by Northern blot analysis. (d) The replicon cells were treated with 100 nM NA255 for 96 h and immunostained with Hoechst 333452 (blue) and anti-NS3 antibody (red). Scale bar, 20 μm. (e) The replicon cells were treated with 100 nM NA255 for the indicated time. The cell lysates were immunoblotted with the indicated antibodies.

RNA-dependent protein kinase[9], 2′-5′-oligoadenylate synthetase[10] or RNase L[11] (data not shown), suggesting that NA255 could be an anti-HCV drug with a previously unknown mode of action.

From a chemical substructure search, we found that NA255 had a structure similar to that of myriocin (2; ref. 12), a selective inhibitor of serine palmitoyltransferase (SPT), the enzyme responsible for the condensation of L-serine with palmitoyl CoA to produce 3-ketodihydrosphingosine, the first step in sphingolipid biosynthesis[13]. We examined the effect of NA255 on SPT inhibitory activity *in vitro* using purified human recombinant SPT encoding two different genes, *LCB1* and *LCB2* (refs. 14,15). NA255 displayed potent inhibition of SPT (IC_{50} = 10 nM; **Fig. 2a**). To assess whether NA255 inhibits the *de novo* biosynthesis of sphingolipids in cells, replicon cells were incubated with [^{14}C]serine in the presence of NA255. NA255 inhibited the *de novo* synthesis of sphingolipids such as [^{14}C]ceramide and [^{14}C]sphingomyelin (SM) in a dose-dependent manner, but no changes were observed in the levels of phosphatidylethanolamine (PE) and phosphatidylserine (PS; **Fig. 2b**). To address whether sphingolipids are required for HCV replication, we attempted knockdown by small interfering RNAs (siRNAs) using two different siRNAs (designated si246 and si633). Immunoblot analysis of extracts from siRNA-transfected replicon cells demonstrated that the LCB1-directed siRNAs effectively reduced expression of LCB1 compared with the control siRNA (**Fig. 2c**). Knockdown of LCB1 substantially inhibited HCV replicon replication, depending on knockdown protein levels of LCB1, and had hardly any effect on cell viability (**Fig. 2d**). To assess whether inhibition of HCV replicon by NA255 was dependent on sphingolipid depletion, we incubated replicon cells with C_2-ceramide, a cell-permeable ceramide analog, in the presence of NA255. Treatment of cells with C_2-ceramide reversed the suppression by NA255 of HCV-NS3 protein levels, in a dose-dependent manner (**Fig. 2e**). Also, sphinganine, a close downstream product of SPT, in combination with NA255 or myriocin, substantially cancelled the replicon inhibitory effect (**Supplementary Table 1** online). To further explore the involvement of the sphingolipid biosynthesis pathway in HCV replication, we examined the effect of sphingolipid-related small molecule compounds on HCV replicon replication. HCV replication was suppressed by myriocin, a known inhibitor of SPT, and fumonisin B1 (3; ref. 16), an inhibitor of dihydroceramide synthase (**Fig. 2f**, upper panels). In mammalian cells, ceramide is synthesized in the endoplasmic reticulum (ER) and translocates to the Golgi compartment for conversion to SM. (1R, 3R)-*N*-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12; 4) is an inhibitor of ceramide trafficking from ER to Golgi[17]. HPA-12 also substantially abrogated HCV replicon replication without cell toxicity (**Fig. 2f**, lower left panel). These results suggested that *de novo* synthesis of sphingolipids is required for HCV replication after translocation to the Golgi, and that interruption of the sphingolipid biosynthesis pathway provides an approach for the development of new HCV therapies.

Recent studies have demonstrated that HCV RNA and NS proteins are associated with intracellular membranes, including ER and Golgi[18], and that the majority of the active replication complexes are present in Golgi-derived detergent-resistant membrane (DRM), most likely in lipid rafts[18,19], microdomains that are enriched in sphingolipids and cholesterol[20]. Since sphingolipids are essential components of the lipid raft, we examined the effect of NA255 on this HCV replication complex formation. When we treated cell lysates with Nonidet P-40, a nonionic detergent, HCV-NS proteins were found in both the DRM and the detergent-sensitive membrane fractions. NA255 treatment led to a marked decrease in the ratio of NS5B proteins in DRM fractions compared with control treatment (**Fig. 3**). We observed a substantial relocation of NS5B at 10 nM NA255 (**Supplementary Fig. 2** online). We obtained a similar result after treating replicon cells with myriocin (**Supplementary Fig. 2** online). In contrast, the DRM fractions of NS3 and NS5A were not affected (**Fig. 3a,b**). Lipid rafts are localized not only in Golgi but also in plasma membrane. To examine the effect of NA255 on the raft-associated protein in host cell plasma membrane, we assessed the distribution of host protein caveolin-2, which is mostly localized in plasma membrane and associated with lipid rafts. Caveolin-2 was present mostly in DRM fractions and was not affected by treatment with NA255 (**Fig. 3a**). These data suggest that NA255 inhibits the interaction of HCV-NS5B with lipid rafts through inhibition of sphingolipid biosynthesis and that this association is involved in HCV replicon replication because an active HCV replication complex is present in Golgi-derived DRM fractions[18,19].

To determine whether HCV protein could interact directly with sphingolipids, we searched for the sphingolipid-binding domain (SBD) in HCV-NS protein. The V3 loop of the human immunodeficiency virus (HIV)-1 surface envelope glycoprotein gp120 is an SBD that mediates the attachment of HIV-1 to plasma membrane lipid rafts[21]. In addition, it has been identified as a common sphingolipid-binding motif in gp120, prion protein (PrP), and β-amyloid peptide[22]. To search for the SBD in HCV protein, we carried out structure

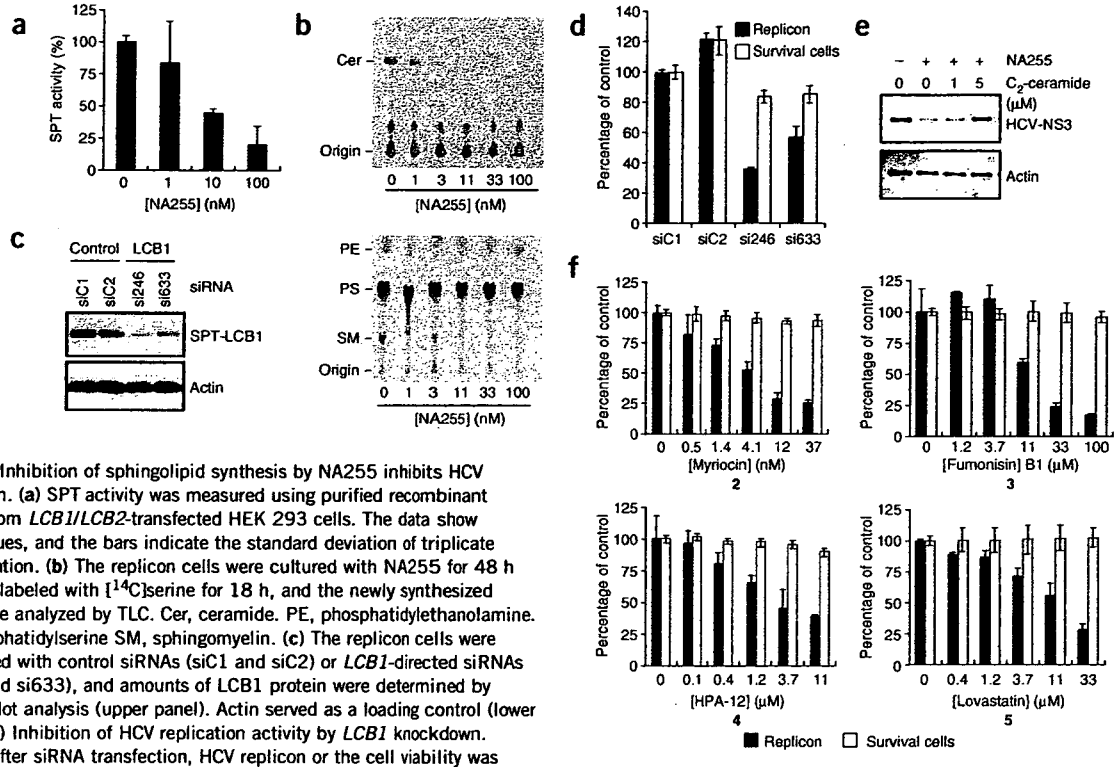

Figure 2 Inhibition of sphingolipid synthesis by NA255 inhibits HCV replication. (**a**) SPT activity was measured using purified recombinant protein from *LCB1*/*LCB2*-transfected HEK 293 cells. The data show mean values, and the bars indicate the standard deviation of triplicate determination. (**b**) The replicon cells were cultured with NA255 for 48 h and then labeled with [^{14}C]serine for 18 h, and the newly synthesized lipids were analyzed by TLC. Cer, ceramide. PE, phosphatidylethanolamine. PS, phosphatidylserine SM, sphingomyelin. (**c**) The replicon cells were transfected with control siRNAs (siC1 and siC2) or *LCB1*-directed siRNAs (si244 and si633), and amounts of LCB1 protein were determined by immunoblot analysis (upper panel). Actin served as a loading control (lower panel). (**d**) Inhibition of HCV replication activity by *LCB1* knockdown. At 96 h after siRNA transfection, HCV replicon or the cell viability was determined by luciferase assay or WST-8 assay. (**e**) The replicon cells were exposed to 1 μM NA255 with indicated concentrations of C_2-ceramide for 96 h. Expression of NS3 was examined with immunoblot analysis (upper panel). Actin served as a loading control (lower panel). (**f**) Replicon cells were treated with the indicated concentrations of myriocin (**2**), fumonisin B1 (**3**), HPA-12 (**4**) or lovastatin (**5**) for 72 h. Replication levels of *HCV* RNA were analyzed by luciferase assay, and cell viability was determined by WST-8 assay. The data represent mean values, and the bars indicate the standard deviation of triplicate determination.

similarity searches using a combinatorial extension program. We found the presence of an HIV-1 gp120 V3-like motif in an HCV-encoded NS5B protein (**Fig. 4a,b**). The V3-like domain of NS5B consists of a helix-turn-helix motif (Glu230–Gly263, **Fig. 4c**) formed by 34 amino acid residues, located in the finger domain of NS5B and of the same size as the V3 loop of gp120, so that superimposition is possible (**Fig. 4b**). It has been demonstrated that SM is associated with a peptide derived from the sphingolipid-binding motif of PrP[22]. To examine whether SM interacts with the SBD of NS5B (NS5B-SBD), we synthesized a peptide (fragment 231–261) derived from the putative sphingolipid-binding motif of NS5B and used surface plasmon resonance (SPR) spectroscopy. We found that SM substantially binds with the NS5B-SBD peptide in a dose-dependent manner, compared with control peptide (**Fig. 4d** and **Supplementary Fig. 3** online). A similar result was observed with a PrP peptide under the same conditions, but it showed weak binding compared with NS5B-SBD (**Fig. 4e**). To confirm the binding of full-length NS5B protein to SM, we evaluated binding by ELISA using lipid-coated microplates. The intact NS5B protein effectively bound to SM, and showed some cross-reaction with galactosylceramide (**Supplementary Fig. 3** online). These results indicate that NS5B has a sphingolipid-binding motif, and the domain was able to directly interact with SM.

Our present study suggests that SPT is a valuable new drug target that can be exploited for the development of HCV therapies. The blocking of SPT activity, both by small molecular weight compounds and by siRNAs, demonstrated notable antiviral effects in replicon cells. In addition, in our preliminary *in vitro* primary hepatocyte infection system, NA255 suppressed HCV replication more continuously than IFN-α (data not shown). This anti-HCV effect is based on the disruption of host sphingolipid biosynthesis. Recently, it was reported that modulation of sphingolipid metabolism affects the susceptibility to HIV-1 infection, thereby inhibiting HIV-1 entry at the plasma membrane[23]. Further studies are needed to address the therapeutic potential of this attractive targeting drug, as current IFN-based HCV therapy has limitations.

HCV RNA replication depends on viral protein association with raft membrane[18,19]. Lipid rafts are built mainly by SM, cholesterol and glycosphingolipids (GSLs). In this report, we suggested that SM is involved in HCV replication, thereby interacting with NS5B. Depletion of cellular cholesterol, another major component of lipid rafts, has recently been shown to reduce HCV RNA replication in HCV replicon cells[18]. We treated replicon cells with statins, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, and found that HCV replication was markedly disrupted by various statins including lovastatin (**5**) in replicon cells (**Fig. 2f**, lower right panel). Recent reports have demonstrated that inhibition of geranylgeranylation, rather than the synthesis of cholesterol itself, is responsible for inhibition of HCV RNA replication[24,25]. To explore the involvement of GSLs in HCV replication, we evaluated replicon activity using an inhibitor of GSL biosynthesis, 1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol (PPMP, **6**). The IC_{50} value of PPMP for inhibition of HCV replication was 1.2 μM,

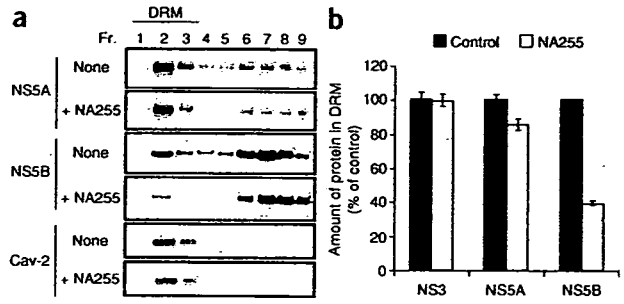

Figure 3 Disruption of the association of HCV -NS5B protein on lipid rafts by NA255. (a) The Replicon cells were treated with 1 μM NA255 for 72 h. Cell lysates were normalized for the amount of NS protein, treated with 1% Nonidet P-40 for 1 h on ice and then subjected to sucrose gradient centrifugation. An aliquot of each fraction was loaded onto gels and analyzed using SDS-PAGE and immunoblotting with the antibodies to NS5A, NS5B and caveolin-2 (Cav-2). Fractions are numbered 1 to 9 in order from top to bottom. (b) Each fraction was diluted by PBS, concentrated and measured by ELISA analysis. The amount of protein in the raft fractions (fractions 1, 2, and 3) was calculated by dividing by the total amount of that protein. The vertical bars represent the standard deviation of triplicate determinations.

whereas PPMP showed cytotoxicity (IC_{50} = 5.3 μM; only fourfold higher than its IC_{50} for inhibition of HCV replication). Furthermore, we found that galactosylceramide has a weak binding activity with NS5B (**Supplementary Fig. 3** online). Thus, GSLs may be involved in the HCV replication process; however, further clarification of the interaction between HCV and raft components is needed. The structures of NS3 protease and NS5B polymerase have previously been determined by X-ray crystallography[26,27]. Here we identified the SBD in NS5B protein but could not find the sphingolipid-binding motif in NS3, suggesting that NS3 could be indirectly associated with lipid rafts through cofactor NS4A[28]. In addition, inhibition of sphingolipid biosynthesis by NA255 disrupted the association of lipid rafts with NS5B, but not with NS3 or NS5A (**Fig. 3b**). Unlike NS3 and NS5A, NS5B seems to have a distinct mechanism of raft association. Host protein hVAP-33 has also recently been implicated in the interaction of NS5B proteins on lipid rafts and HCV replication[28]. Detailed descriptions of raft-protein interactions will provide new therapeutic strategies for rational drug design. Using point mutation analysis of NS5B-SBD, we are conducting ongoing studies to clarify the link between host sphingolipids and HCV replication competency.

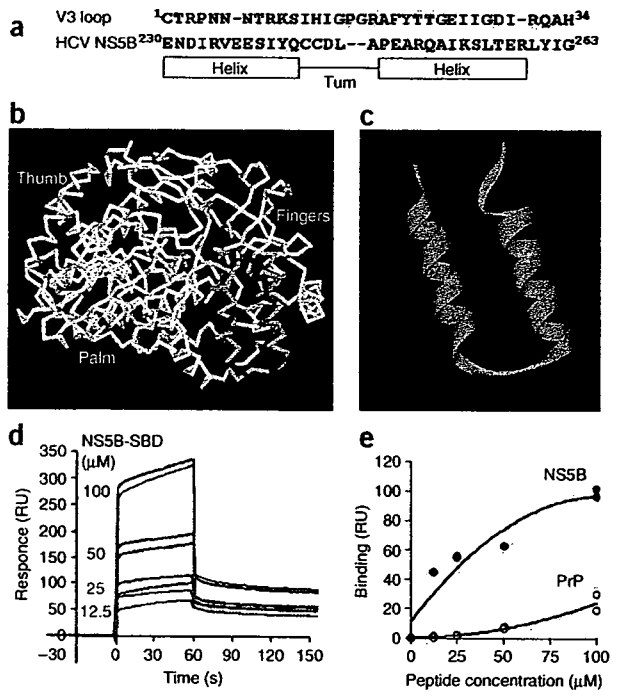

Figure 4 The V3-like region of HCV-NS5B is a sphingolipid-binding domain. (a) Structure-based sequence alignment of HCV-NS5B and HIV-1 gp120 (V3 loop). (b) Overall structure of HCV-NS5B and the V3 loop region of HIV-1 gp120. Structure similarity searches were performed using the two-chain calculation routine of the combinatorial extension program. The putative sphingolipid-binding motif in Glu230–Gly263 of NS5B (yellow) and the V3 loop of HIV-1 gp120 (blue). See Methods for database accession codes. (c) Ribbon diagram of the structure of the putative NS5B sphingolipid-binding domain. (d) Sensogram for interaction of SM with putative sphingolipid-binding motif peptide of NS5B monitored by SPR. See **Supplementary Methods**. (e) Binding of SM to sphingolipid-binding motif peptides of PrP or NS5B. The binding levels (RU value at 80 s) were obtained directly from the sensogram after subtraction of the background signal in the absence of peptide.

METHODS

Isolation of NA255. Strain F1476, a producer strain of NA255, is a filamentous fungus that was isolated from fallen leaves collected in Kamakura, Japan. Strain F1476 was identified as *Fusarium* sp. One loopful of microorganisms obtained from a slant culture of strain F1476 was inoculated into Erlenmeyer flasks with baffles containing liquid media (2% glucose, 1.5% glycerol, 1% potato starch, 0.25% polypeptone, 0.35% yeast extract, 0.5% calcium carbonate, 0.3% sodium chloride, 0.005% zinc sulfate heptahydrate, 0.0005% copper sulfate pentahydrate, 0.0005% manganese sulfate tetrahydrate and 1% toasted soya); cultures were then incubated, shaking, at 25 °C for 3 d to obtain an inoculated culture seed. This culture seed was inoculated into Erlenmeyer flasks with baffles containing solid media (40 g pressed barley, 24 ml SF1 solution (0.1% yeast extract, 0.05% sodium tartrate, 0.05% potassium dihydrogen phosphate)), followed by stationary culturing at 25 °C for 11 d. *n*-Butanol (12.5 l) was then added to the culture, the culture was let stand overnight, and then the culture was filtered to obtain an *n*-butanol extract. After concentrating, the extract was suspended in 1 l of water, adjusted to pH 2 with hydrochloric acid, and was extracted with 1.1 l of ethyl acetate. The aqueous layer was extracted again with 1.1 l of ethyl acetate and combined with the first extract. Water (0.9 l) was then added to the ethyl acetate extract (2.2 l) and distributed after adjusting to pH 10 with an aqueous sodium hydroxide solution. Ethyl acetate (1 l) was again added to the resulting aqueous layer and then extracted after adjusting to pH 3 with hydrochloric acid. The resulting aqueous layer was again extracted with 1 l of ethyl acetate. The ethyl acetate extract (2 l) thus obtained was then dried over sodium sulfate followed by concentrating and drying to obtain 567 mg of crude extract. This crude extract was dissolved in methanol and repeatedly purified by HPLC (CCPP-D, MCPD-3600 System (Tosoh), CAPCELL PAK C18 (UG 80, 20 mm × 250 mm, Shiseido)), using water containing 0.01% trifluoroacetic acid and acetonitrile containing 0.01% trifluoroacetic acid (15% acetonitrile to 98% acetonitrile, stepwise). NA255 was concentrated under reduced pressure to obtain 380 mg of NA255 in the form of a white powder. ^{1}H-NMR (in methanol d-4): δ 0.89 (t, J = 7 Hz, 3H), 1.20–1.40 (m, 14H), 1.53 (m, 4H), 1.73 (s, 3H), 1.77 (s, 3H), 1.96 (m, 2H), 2.42 (m, 4H), 2.57 (d, J = 16.5 Hz, 1H), 2.89 (d, J = 16.5 Hz, 1H), 2.91 (dd, J = 14, 9 Hz, 1H), 3.15 (dd, J = 14, 4.5 Hz, 1H), 3.20 (d, J = 8 Hz, 1H), 4.47 (d, J = 6 Hz, 2H), 4.63 (dd, J = 9, 4.5 Hz, 1H), 5.43 (m, 1H), 5.52 (m, 2H), 6.78 (d, J = 9 Hz, 2H), 7.10 (d, J = 9 Hz, 2H); FAB-MS (*m/z*; positive mode; matrix m-NBA): 660 $(M + H)^+$; FAB-MS (*m/z*; negative mode; matrix m-NBA): 658 $(M - H)^-$.