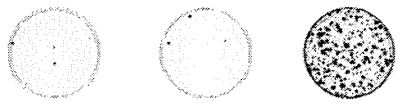
A



B

Replicon cell clone	Nucleotide mutation	Amino acid substitution	Affected protein	Replicon RNA amounts (copies/μg RNA)
1	5272 (6932) G→A	2198 R→H	NS5A	2.1×10^8
2	7362 (9022) A→G	2895 R→G	NS5B	9.9×10^7
3	2536 (4196) C→T	1286 T→I	NS3	9.5×10^7
4	2536 (4196) C→T	1286 T→I	NS3	9.9×10^7
5	2536 (4196) C→T	1286 T→I	NS3	5.6×10^7
6	2536 (4196) C→T	1286 T→I	NS3	4.0×10^7
7	2536 (4196) C→T	1286 T→I	NS3	8.3×10^7
8	2536 (4196) C→T	1286 T→I	NS3	8.7×10^7
9	6166 (7826) C→T	2496 T→I	NS5B	9.3×10^7
	7363 (9023) G→A	2895 R→K	NS5B	
10	5241 (6901) A→G	2188 T→A	NS5A	5.1×10^7

C

Replicon constructs	Protein	Colony formation efficiency (cfu/μg RNA)	Transient replication efficiency (72h/4h†)
SGR-S310/A	None	0.4×10^0	4.4×10^{-3}
T1286I	NS3	2.9×10^3	6.5×10^{-3}
T2188A	NS5A	2.3×10^2	4.9×10^{-3}
R2198H	NS5A	3.7×10^3	6.0×10^{-2}
S2210I	NS5A	6.2×10^2	1.1×10^{-2}
T2496I	NS5B	0.2×10^0	5.0×10^{-3}
R2895K	NS5B	2.3×10^3	7.9×10^{-2}
R2895G	NS5B	9.8×10^2	2.6×10^{-2}
T2496I+R2895K	NS5B	4.1×10^3	2.6×10^{-1}
Con1-NK5.1	NS3+NS5A	9.1×10^2	6.3×10^0
N	None	NA††	7.1×10^{-2}
SGR-JFH1	None	5.3×10^3	1.5×10^0

Figure 1. S310 subgenomic replicon analysis. (*A*) Three million HuH-7 cells were electroporated with 10 μg RNA from SGR-S310/A or SGR-S310/B or 0.1 μg RNA from SGR-JFH1. G418-selected colonies were fixed and stained after 3 weeks. (*B*) Non-synonymous mutations identified in the replicon genomes and HCV RNA titers in the replicon clones. Nucleotide positions within the S310 subgenomic replicon and within the full-length S310 genome (in parentheses) are given. (*C*) Replication potential of the adaptive mutants as determined by the colony-formation assay using Neo-replicons and by the transient replication assay using Fluc-replicons. †72 h/4 h, transient replication efficiency was determined as a ratio of luciferase activity in the transfected cells between 72 h and 4 h post transfection. ††NA, not available.

clones); T2188A or R2198H in NS5A (2 clones); an R2895G substitution in NS5B (1 clone); and T2496I in NS5A plus R2895K in NS5B (1 clone). These mutations and the S2210I mutation (corresponding to S2204I in genotype 1 replicon)[7,8] were introduced, individually or in combination, into the parental SGR-S310 and the colony-formation efficiencies of the mutant replicons were tested. All mutations, except T2496I, increased the colony formation, indicating an adaptive phenotype (Figure 1*C*, Supplementary Figure 3*B*). Transient replication efficiency was also tested using firefly luciferase reporter replicons. SGR-S310/Luc did not replicate in Huh-7.5.1 cells, whereas the adaptive mutants displayed varying degrees of replication (Figure 1*C*, Supplementary Figure 3*C*). Adaptive mutations T2496I and R2895K, when combined together, most efficiently enhanced the colony formation as well as transient replication (Figure 1*C*). Interestingly, T1286I and R2895G found in our study correspond to the Con1 adaptive mutations T1280I and R2884G, respectively.[11,12] T2188A or R2198H in NS5A were identified in 2 replicon clones and are located close to S2210I. Indeed, S2210I also enhanced SGR-S310 replication, suggesting that this region might be important for HCV replication. S310 replicons with adaptive mutations were compared with genotype 1b (Con1 and N) and 2a (JFH-1) replicons. Colony-formation efficiencies of most S310 adaptive replicons were at levels comparable with Con1 and JFH-1 (Figure 1*C*, Supplementary Figure 3*B*). In contrast, S310 adaptive replicons replicated less efficiently than Con1-NK5.1 and JFH-1 replicons in transient replication assays. However, genotype 1b N replicon replicated at a level similar to some S310 adaptive replicons (Figure 1*C*, Supplementary Figure 3*C*). Future studies will dissect the detailed mechanisms that underlie the effects of these mutations.

Successful generation of a genotype 3a replicon provided a unique opportunity to compare the susceptibility of genotype 3a (SGR-S310), 1b (Con1[13]), and 2a (JFH-1/4-1[13]) replicons to HCV inhibitors. Interferon-alfa dose-dependently decreased the replication of all tested genotypes (Figure 2*A*), whereas a protease inhibitor, BILN-2061, was more effective against replicons from genotypes 1b and 2a than 3a (Figure 2*B*). The non-nucleoside polymerase inhibitor JTK-109 was more potent against genotype 1b and 3a (Figure 2*C*). However, the nucleoside polymerase inhibitor, PSI-6130, equally inhibited all genotypes (Figure 2*D*).

In conclusion, we established a subgenomic replicon for genotype 3a, which should be useful for understanding the specific characteristics of this genotype and for the screening of antiviral chemicals that are effective against this genotype. Construction of a full-length infectious S310 clone is in progress.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.09.017.

References

1. Di Bisceglie AM. Hepatology 1997;26(Suppl):34S–38S.
2. Lauer GM, et al. N Engl J Med 2001;345:41–52.
3. Ohno T, et al. J Clin Microbiol 1997;35:201–207.
4. Hui JM, et al. J Gastroenterol Hepatol 2002;17:873–881.
5. Gottwein, JM et al. Gastroenterology 2011;141:1067–1079.
6. Lohmann, V et al. Science 1999;285:110–113.
7. Blight KJ, et al. Science 2000;290:1972–1974.
8. Blight KJ, et al. J Virol 2003;77:3181–3190.
9. Kato T, et al. Gastroenterology 2003;125:1808–1817.
10. Kato T, et al. J Med Virol 2001;64:334–339.
11. Krieger N, et al. J Virol 2001;75:4614–4624.
12. Lohmann V, et al. J Virol 2001;75:1437–1449.

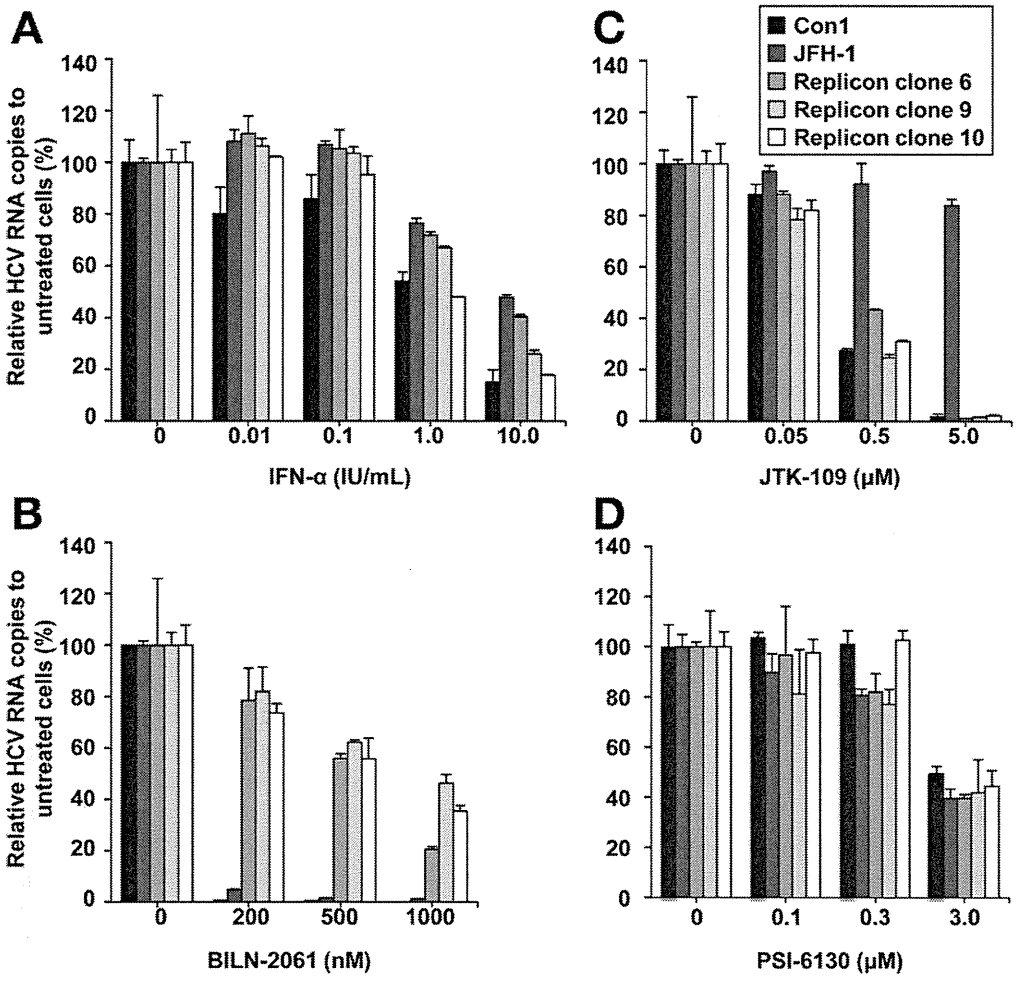


Figure 2. Effects of antiviral inhibitors on the replication of S310 subgenomic replicons. Three cell clones (clone 6, 9 and 10) carrying genotype 3a S310 replicons and one cell clone each harboring genotype 1b Con1 and genotype 2a JFH-1 replicons were treated with the indicated concentrations of (A) interferon alpha, (B) HCV protease inhibitor BILN-2061, (C) the non-nucleoside polymerase inhibitor JTK-109, and (D) the nucleoside polymerase inhibitor PSI 6130 for 72 hours and replication levels were measured by quantifying intracellular HCV RNA. Results are means ± standard deviations of 3 replicates.

13. Miyamoto M, et al. Intervirology 2006;49:37–43.

Received February 22, 2012. Accepted September 10, 2012.

Reprint requests

Address requests for reprints to: Takaji Wakita, MD, PhD, Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. e-mail: wakita@nih.go.jp; fax: +81-3-5285-1161.

Acknowledgments

Huh-7.5.1 cells were kindly provided by Dr Francis V. Chisari. BILN-2061, JTK-109, and PSI-6130 were generous gifts from Boehringer Ingelheim (Canada) Ltd., Japan Tobacco, Inc., and Pharmasset, Inc., respectively. Con1-NK5.1 and N replicon constructs were kindly provided by Dr Ralf Bartenschlager and Dr Stanley Lemon, respectively. We thank Dr Hideki Aizaki and Dr Hussein H. Aly for their helpful discussions. We also thank Ms Minako Kaga for her technical assistance.

DDBJ/EMBL/GenBank accession numbers: S310/A: AB691595, S310/B: AB691596, SGR-S310/A: AB691597, SGR-S310/B: AB691598, SGR-S310/Luc: AB691599.

Dr Saeed is presently at the Center for the Study of Hepatitis C, The Rockefeller University, New York, NY.

Dr Suzuki is presently at Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan.

Conflicts of interest

The authors disclose no conflicts.

Funding

This work was partially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan, from the Ministry of Education, Culture, Sports, Science and Technology, from the National Institute of Biomedical Innovation, and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation and from the French Agence Nationale de la Recherche sur le SIDA et les Hépatites Virales (ANRS, contract 07306).

Supplementary Methods

Cell Culture

The human hepatoma cell line HuH-7[1] and its derivative cell line Huh-7.5.1[2] were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a 5% CO_2 incubator.

Primary human hepatocytes (PHH) were isolated from an encapsulated liver sample.[3] Isolated PHH were seeded in 12-well plates and cultured at 37°C in Lanford medium before infection.

PHH Infection With HCV-Positive Sera

Three days post seeding, PHH were inoculated with HCV-positive sera. After 16 h of inoculation, monolayers were washed with William's E medium and fresh Lanford medium was added. Cells were harvested at 72 h post infection. Total RNA was isolated using a guanidinium isothiocyanate solution (RNAble; Eurobio, Courtaboeuf, France) and intracellular levels of HCV RNA were quantified using the SuperScript III Platinium One-Step quantitative reverse transcription polymerase chain reaction (RT-PCR) system (Invitrogen, Carlsbad, CA) and a LightCycler480 real-time PCR system (Roche Diagnostics, Meylan, France).

HCV Genotype 3a Clone

Clone S310 was isolated from a 71-year-old female patient suffering from post liver transplantation HCV recurrence. She was diagnosed with HCV genotype 3a infection at the age of 59 years and underwent liver transplantation 4 years later due to liver cirrhosis. HCV-RNA titer was 2.8×10^6 copies/mL. Total RNA extracted from 100 μL serum using the acid-guanidinium isothiocyanate-phenol-chloroform method (Isogen-LS; Nippon Gene, Tokyo, Japan) was precipitated with isopropanol, washed with ethanol, and dissolved in 10 μL nuclease-free water. An aliquot of 4 μL was subjected to reverse transcription using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript III; Invitrogen) at 42°C for 50 min and then at 50°C for 10 min.

Isolation of HCV

The sequences of 4 isolates of genotype 3a (accession numbers AF046866, D28917,[4] X76918, and D17763[5]) that were obtained from the HCV database (http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html) were aligned and PCR primers were designed based on the conserved sequences. These primers were used to amplify the complementary DNA (cDNA) of S310 into 9 overlapping fragments by nested PCR (nt 1–370, nt 127–1284, nt 1117–1997, nt 1704–3352, nt 3152–5080, nt 4869–6842, nt 6601–8129, nt 7988–9145, and nt 9082–9576; nucleotide numbers refer to the positions on S310, with nt 1 being the first nucleotide of the 5′ UTR). The sequence of these primers is shown in Supplementary Table 1. Two microliters of cDNA was subjected to PCR using Pyrobest DNA polymerase (Takara Bio, Kyoto, Japan) and the outer set of primers, and this first-round PCR product (2 μL) was further amplified by a second round of PCR using the inner set of primers. PCR conditions for the first and second rounds of PCR consisted of 35 cycles each of denaturation at 98°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min. A fragment encompassing the 5′ end of the viral genome (nt 1–370) was amplified by 5′RACE. Briefly, cDNA was synthesized with a 5′ UTR primer (antisense), tailed with a dCTP homopolymer by using terminal deoxynucleotidyl transferase, and amplified by PCR (5′ RACE System for Rapid Amplification of cDNA Ends; Invitrogen) using TaKaRa LA Taq polymerase (Takara Bio). The PCR products of all fragments were separated by agarose gel electrophoresis, cloned into the pGEM-T EASY vector (Promega, Madison, WI) and sequenced using the Big Dye Terminator Mix and an automated DNA sequencer. The consensus sequence of 5 to 9 isolated cDNA clones was adopted for each fragment. Two major populations of the virus were identified in the patient's serum that differed in 4 amino acids in the NS3 protein (aa 1039, 1183, 1463, and 1504), and these populations were designated as S310/A and S310/B (DDBJ/EMBL/GenBank accession number: AB691595 and AB691596, respectively). To assess the complexity of the HCV population in the patient's serum, the hypervariable region sequences of 10 clones were determined.

Computer Analysis

A phylogenetic tree was constructed using the neighbor-joining method to examine the relationship between the polyprotein region of S310 and that of other HCV genotype 3a isolates available in the database. In order to analyze the diversity in each subgenomic region, the genetic distance was calculated between all possible pairs of genotype 3a isolates and between S310/A and other isolates using MacVector software (MacVector, Inc., Cary, NC). The ratios of these 2 values (mean genetic distance between S310/A and other isolates/mean genetic distance among all genotype 3a isolates) were compared.

Construction of Replicons

Based on the consensus sequence of S310, we assembled pS310/A and pS310/B, which contained the full-length S310/A and S310/B cDNA, respectively, downstream of the T7 RNA polymerase promoter. Briefly the 9 amplicons described here were combined by overlapping PCR and ligated with pGEM-T EASY vectors to generate 6 plasmids (A through F) in such a way that each plasmid contained a unique restriction enzyme

cleavage site toward the 3′ end of the viral fragment, which overlapped with the 5′ end of the next fragment. For this purpose, we took advantage of the EcoRl restriction site that is present in the polycloning site of the plasmid toward the 5′ end of the viral fragment. Plasmid A contained the T7 promoter sequence followed by one G-nucleotide and nt 1–3352 of S310, while plasmids B, C, D, and E contained nt 1704–4307, nt 4044-6013, nt 5424–7755, and nt 7276–9425, respectively. Plasmid F contained the fragment constructed by combining the C-terminal end of NS5B (nt 9182–9402) and the variable and poly U/UC regions of the S310/A 3′UTR (nt 9403–9610) with the last 44 nucleotides of JFH-1. Restriction sites for EcoRl and Xbal were introduced upstream of the T7 promoter sequence and downstream of the conserved region, termed the *X-region*, of the 3′UTR, respectively, and the restriction sites of these enzymes that were present within the cDNA were removed by PCR-based mutagenesis. In the neomycin-based subgenomic replicons (SGR-S310/A and SGR-S310/B, accession number: AB691597 and AB691598, respectively), the cassette containing the neomycin phosphotransferase gene and the EMCV IRES replaced the region of S310 that encompasses amino acids 20–1032. Firefly luciferase–based subgenomic replicons (SGR-S310/Luc, accession number: AB691599) were generated from SGR-S310/A by replacing amino acids 20–1032 of S310/A with the cassette containing firefly luciferase and the EMCV IRES from pSGR-JFH1/Luc.[6]

RNA Synthesis

RNA was synthesized by in vitro transcription as described previously.[7] Briefly, the plasmids carrying the cDNA described here were linearized with the XbaI restriction enzyme and 5′ overhangs were removed by treating with mung bean nuclease. Reaction mixtures were further incubated at 50°C for 1 h with 2 μL 20 mg/mL proteinase K and 10 μL 10% sodium dodecyl sulfate to degrade nucleases, and templates were purified with 2 rounds of phenol-chloroform extraction and ethanol precipitation. Three micrograms of templates were subjected to in vitro transcription using a MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Synthesized RNA was treated with DNase I (Ambion) and then purified using ISOGEN-LS (Nippon Gene). The quality of the synthesized RNA was examined by agarose gel electrophoresis.

RNA Transfection

In vitro transcribed RNA or total cellular RNA isolated from replicon cells was introduced into cells by electroporation. Trypsinized cells were washed twice with serum-free Opti-MEM I (Invitrogen) and 3.0×10^6 cells were resuspended in 400 μL cytomix buffer.[8] RNA was delivered into cells by a single pulse of 260 V and 950 μF using the Bio-Rad Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately suspended in culture medium and transferred to the appropriate plates. For G418 selection of colonies, the transfected cells were seeded in 10-cm dishes, each containing 8 mL culture medium. G418 (500 μg/mL; Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 24 h after transfection. Culture medium supplemented with G418 was replaced every 3 days. Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet or replicon colonies were picked and expanded.

Analysis of G418-Resistant Cells

G418-resistant colonies were collected and used for further analysis. Colonies were independently isolated using cloning cylinders (Asahi Techno Glass Co., Tokyo, Japan) and were expanded until they were 80%–90% confluent in 10-cm dishes. Expanded cells were harvested for nucleic acid and protein analysis. Total RNA was isolated from the cells using the ISOGEN reagent (Nippon Gene). Another aliquot of the cell pellet was dissolved in RIPA buffer containing 0.1% sodium dodecyl sulfate for Western blot analysis. For immunofluorescence analysis of viral proteins, cells were seeded on 12-well slides.

Quantification of HCV RNA by Real-Time RT-PCR

Copy numbers of HCV RNA were determined by real-time detection RT-PCR, as described previously,[9] using the ABI Prism 7700 Sequence Detector System (Applied Biosystems Japan, Tokyo, Japan). The concentration of total RNA in the cells was determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockford, IL).

Northern Blot Analysis

Isolated RNAs (3 μg) from replicon cells were separated on a 1% agarose gel containing formaldehyde, transferred to a positively charged nylon membrane (Hybond-N+; GE Healthcare UK Ltd., Buckinghamshire, UK) and immobilized using a FUNA-UV-LINKER (Funakoshi, Tokyo, Japan). Hybridization was carried out with a [α-^{32}P]dCTP-labeled DNA probe using Rapid-Hyb buffer (GE Healthcare UK Ltd.). The DNA probe was synthesized from a BsrGI-MfeI fragment of the S310 clone that contained NS3-5B genes using the Megaprime DNA labeling system (GE Healthcare UK Ltd.).

Indirect Immunofluorescence

Untransfected HuH-7 cells or S310 replicon-replicating cells were grown on a glass slide for 24 h and fixed in acetone-methanol (1:1 [vol/vol]) for 10 min at −20°C. Cells were then incubated in immunofluorescence buffer (phosphate-buffered saline, 1% bovine serum albumin, 2.5 mM EDTA). S310 patient serum was added at

a dilution of 1:200 in immunofluorescence buffer. After incubation for 1 h at room temperature, cells were washed and then incubated with an Alexa Fluor488—conjugated goat anti-human IgG antibody (Invitrogen) in immunofluorescence buffer. The glass slide was washed and a cover glass was mounted using PermaFluor mounting solution (Thermo Scientific, Cheshire, UK). Cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis of HCV Proteins

The protein samples were separated on 12.5% polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). Transferred proteins were incubated with 2% skim milk. Anti-NS3 mouse monoclonal antibody (clone 8G2, Abcam, Cambridge, UK) and peroxidase-labeled sheep anti-mouse IgG (Cell Signaling Technology, Danvers, MA) were used to detect HCV proteins. The signals were detected with a chemiluminescence system (ECL Prime; GE Healthcare UK Ltd.).

Identification of Mutations

cDNA was synthesized from total RNA that was extracted from replicon-expressing cells at 2 different times. These cDNAs were amplified into 5 overlapping fragments that spanned the 5′UTR and the NS3-NS5B region using LA Taq DNA polymerase (Takara Bio) and the primers described in Supplementary Table 1. The sequence of each amplified DNA was determined. The mutations identified were subsequently introduced into SGR-S310/A and SGR-S310/Luc by PCR-mediated mutagenesis.

Luciferase Assay

Five micrograms of RNA, prepared by in vitro transcription of S310/SG-FLuc constructs with or without adaptive mutations, were introduced into 3.0×10^6 Huh-7.5.1 cells by electroporation. Cells were harvested with Cell Culture Lysis Reagent (Promega) at 4, 24, 72, and 96 h post electroporation, and luciferase activity was determined by use of a Luciferase Assay System (Promega) and the Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany).

Inhibition of S310 Replicon Replication by Specific Inhibitors

S310 replicon cell clones 6, 9, and 10 and the genotype1b Con1 and 2a JFH-1 replicon cells,[10] were seeded into 24-well plates at a density of 5.0×10^4 cells/well. On the next day, the culture medium was replaced with medium containing 0.1% dimethyl sulfoxide with or without various concentrations of interferon alfa (Dainippon-Sumitomo, Osaka, Japan), the specific NS3 protease inhibitor, BILN-2061 (Boehringer Ingelheim Ltd., Québec, Canada), or the NS5B inhibitors, JTK-109 (Japan Tobacco, Inc., Osaka, Japan) and PSI-6130 (Pharmasset, Inc., Princeton, NJ). After 72-h incubation, cells were harvested and HCV RNA was quantified as described.

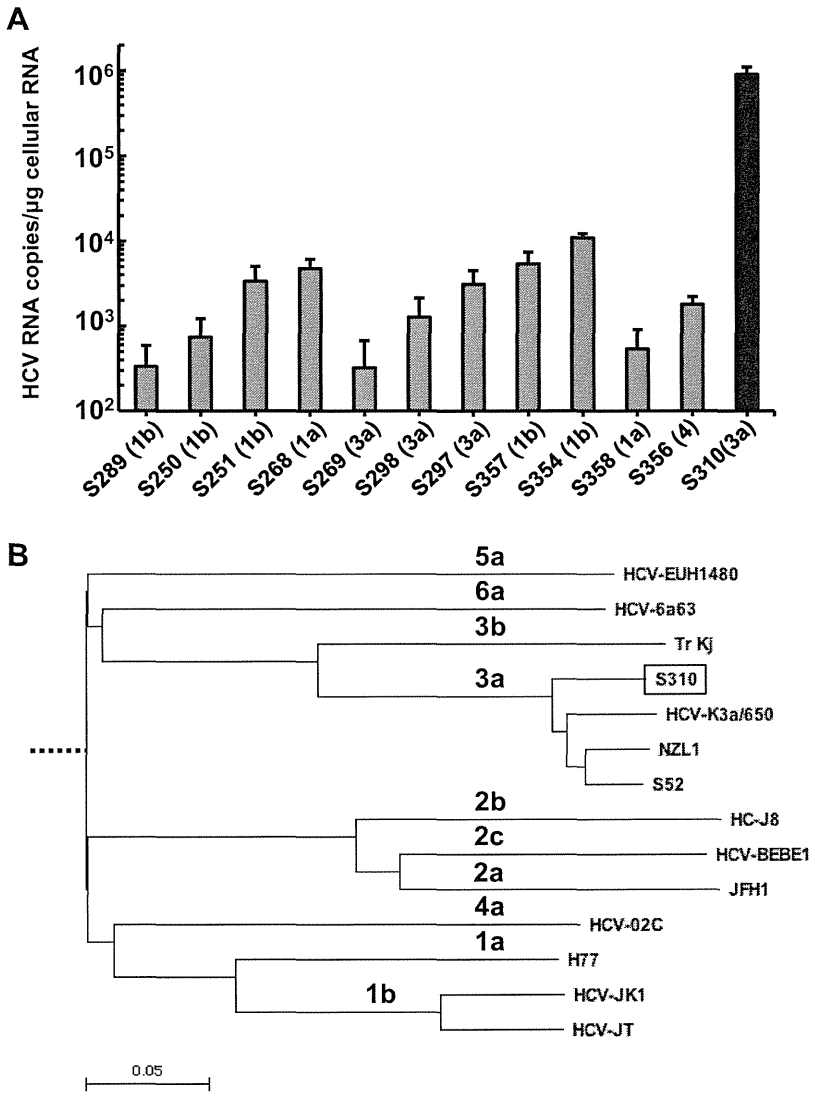
Supplementary References

1. Nakabayashi H, et al. Cancer Res 1982;42:3858–3863.
2. Zhong J, et al. Proc Natl Acad Sci U S A 2005;102:9294–9299.
3. Pichard L, et al. Methods Mol Biol 2006;320:283–293.
4. Yamada N, et al. J Gen Virol 1994;75:3279–3284.
5. Sakamoto M, et al. J Gen Virol 1994;75:1761–1768.
6. Kato T, et al. J Clin Microbiol 2005;43:5679–5684.
7. Wakita T, et al. Nat Med 2005;11:791–796.
8. van den Hoff MJ, et al. Nucleic Acids Res 1992;20:2902.
9. Takeuchi T, et al. Gastroenterology 1999;116:636–642.
10. Miyamoto M, et al. Intervirology 2006;49:37–43.

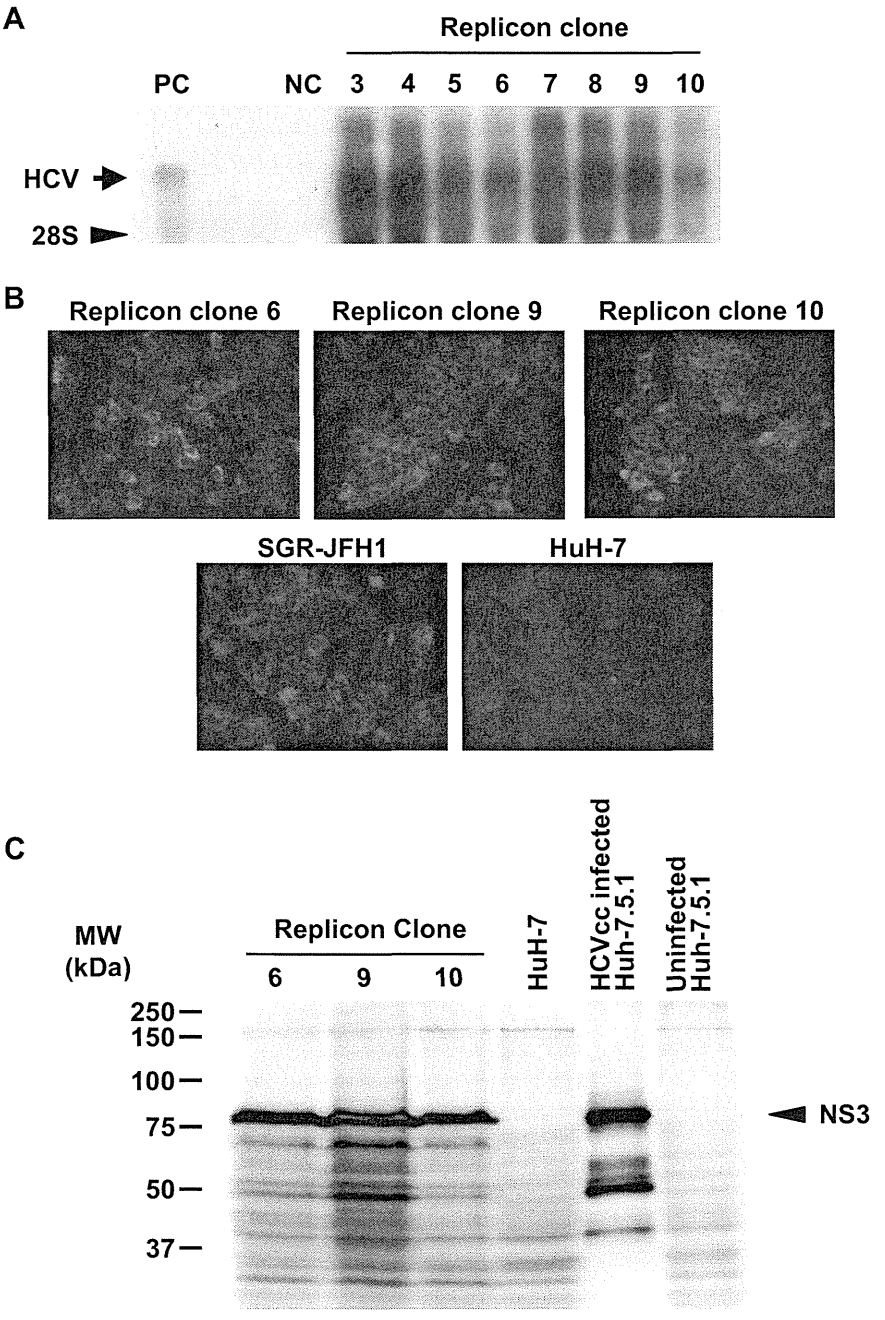
Supplementary Table 1. Primers for Amplification of the S310 HCV Strain

	Fragment		Primer sequence (5′→3′)
1 (5′ RACE)[a]	Outer	Antisense	CTTGACGTCCTGTGGGCGA
	Inner	Antisense	TTTTTCTTTGGGGTTTAGG
2	Outer	Sense	GTCTTCACGCGGAAAGCGC
		Antisense	CACCCAAACCACCGACCAC
	Inner	Sense	CCGGGAGAGCCATAGTGGTC
		Antisense	TCCTGAAAGATGGCCTGGGTA
3	Outer	Sense	CTTGGCCCCTCTATGGTAA
		Antisense	GATGTTTCCTGAAGCAGTCG
	Inner	Sense	AGTCATGTGGACCTATTAGT
		Antisense	CACCCAAACCACCGACCAC
4	Outer	Sense	ATGGCTCGTGGCACATCAA
		Antisense	TAGTCATCAGCAGGTCCCAA
	Inner	Sense	GCTCAGCAGCTGCAAGCCCAT
		Antisense	CGCAAAGAATATCTCCGCAAG
5	Outer	Sense	ATTTTTGACATCACTAAGCTAC
		Antisense	AGTGTTGGCTTAAGCCGCA
	Inner	Sense	AATACTTCCAGATGATCATACT
		Antisense	GTGACAGAAAGTGGGCAT
6	Outer	Sense	GTTTCCCGCAGCCAACGT
		Antisense	GTCTCTCAACATCGAGGT
	Inner	Sense	CGGTGAAAGACCGTCTGGA
		Antisense	CAGGGGAGTTGAGATCCT
7	Outer	Sense	GGCCGCGTACATGTGCTAAC
		Antisense	CCGCAGACAAGAAAGTCCGGGT
	Inner	Sense	CTATGGCGCGTGGCTGCCA
		Antisense	ACCCCCAGGTCAGGGTACAC
8	Outer	Sense	CATAACCTAGTCTATTCAACG
		Antisense	TGGTCTTGGTGCGTACCG
	Inner	Sense	GCTCCGTCTGGGAGGACTTGC
		Antisense	CTCGTGCCCGATGTCTCCAA
9	Outer	Sense	TGCTCCTCCAACGTCTCCGT
		Antisense	GCGGCTCACGGACCTTTCAC
	Inner	Sense	GTCGCGGGGACACTCAGGAA
		Antisense	ACTAGGGCTAAGATGGAGCC

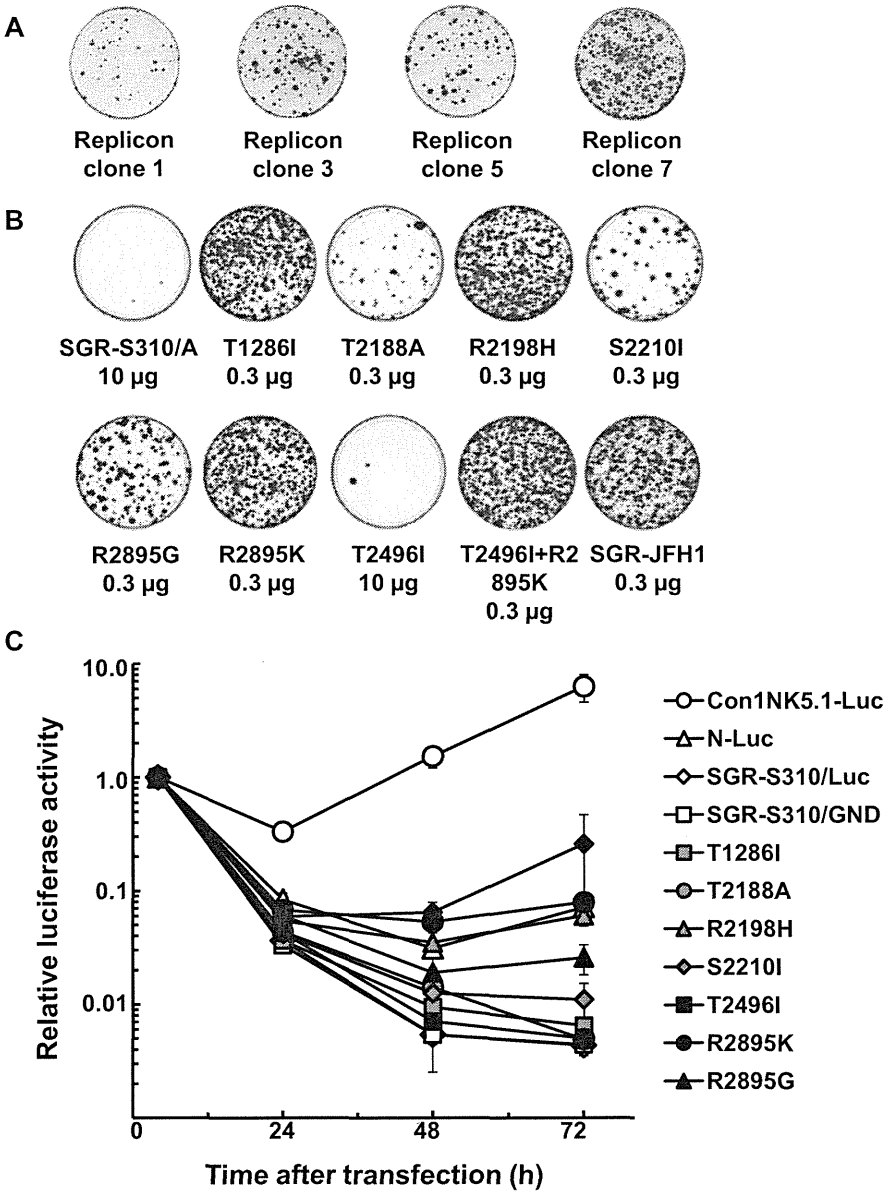
RACE, rapid amplification of complementary DNA ends.
[a]Forward primers used were those in the 5′RACE kit (Abridged Universal Amplification Primer (AUAP) for the first round of PCR and Universal Amplification Primer (UAP) for the second round of PCR).



Supplementary Figure 1. Infection of PHH with HCV patient sera and phylogenetic tree analysis of the S310 strain. (*A*) PHH were exposed to sera of patients infected with genotype 1b (S289, S250, S251, S357, S354), 1a (S268, S358), 3a (S269, S298, S297, S310), and 4 (S356) for 16 h (25 µL/well, except for S310, 10 µL). Intracellular HCV RNA was quantified 72 h post inoculation. Experiments were done in triplicate and data are presented as means ± standard deviation. (*B*) The phylogenetic tree was constructed using the polyprotein region of S310 and HCV strains of different genotypes. The HCV strains analyzed and their corresponding GenBank accession numbers are: K3a/650; D28917, NZL1; NC_009824, S52; GU814263, EUH1480, HCV-6a63; DQ480514, Tr Kj; D49374, HC-J8; D10988, BEBE1; D50409, JFH-1; AB047639, HCV-02C; DQ418784, H77; AF009606, HCV-JK1; X61596 and HCV-JT; D11168. The root of the tree was tentatively taken as the midpoint of the longest path. The length of the horizontal bar indicates the number of nucleotide substitutions per site.



Supplementary Figure 2. Detection and quantification of HCV RNA and proteins in replicon cells. (*A*) Total RNA (3 μg) from replicon cells was analyzed by Northern blot; 5.0 × 10^7 copies of in vitro–transcribed RNA were loaded in parallel as a positive control (PC), while total RNA from untransfected HuH-7 cells served as the negative control (NC). Replicon RNA was detected using a [α-^{32}P]dCTP-labeled DNA probe. Arrow and arrowhead indicate the positions of the replicon RNA and 28S ribosomal RNA, respectively. (*B*) Subcellular localization of viral proteins determined by immunofluorescence. S310 replicon cell clones, JFH-1 replicon cells, and untransfected HuH-7 cells were grown on glass slides for 24 h. After fixation, cells were incubated with patient serum. (*C*) Western blot analysis. Cell lysates were prepared from replicon clones 6, 9, and 10, untransfected HuH-7, and HCVcc (J6/JFH1)-infected Huh-7.5.1 cells and uninfected Huh-7.5.1 cells. Protein (10 μg) was resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and viral nonstructural protein NS3-specific bands were detected using an anti-NS3 mouse monoclonal antibody (clone 8G2). Arrow indicates the position of NS3.



Supplementary Figure 3. Analysis of the effect of mutations on the colony-forming efficiency and transient replication of the subgenomic replicon S310. (*A*) Total RNA was isolated from the indicated replicon cell clones and 10 μg RNA was introduced into 3 million naïve HuH-7 cells by electroporation. After 3 weeks of G418 selection (500 μg/mL), colonies were stained. (*B*) Three million HuH-7 cells were electroporated with the indicated amounts of transcribed RNA and colonies were selected by a 3-week G418 selection. The JFH-1 subgenomic RNA was included as a positive control. (*C*) Huh-7.5.1 cells were transfected with the transcribed RNA from pSGR-S310/Luc and pSGR-S310/Luc constructs with mutations (GND mutation in NS5B, T1286I, T2188A, R2198H, S2210I, T2496I, R2895K, R2895G, and T2496I+R2895K) and Con1-NK5.1/Luc and N/Luc replicon. Transfected cells were harvested at the indicated time points and at 4 h post transfection. Relative luciferase activity (arbitrary units) was measured in the cell lysate and was normalized to the activity at 4 h post transfection. Assays were performed in triplicate, and data are presented as means ± standard deviation.

ELSEVIER

Institut Pasteur

Microbes and Infection 15 (2013) 45–55

www.elsevier.com/locate/micinf

Original article

Selective estrogen receptor modulators inhibit hepatitis C virus infection at multiple steps of the virus life cycle

Yuko Murakami [a,*], Masayoshi Fukasawa [b], Yukihiro Kaneko [a], Tetsuro Suzuki [c,1], Takaji Wakita [c], Hidesuke Fukazawa [a]

[a] *Department of Bioactive Molecules, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo, Japan*
[c] *Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan*

Received 15 June 2012; accepted 13 October 2012
Available online 23 October 2012

Abstract

We screened for hepatitis C virus (HCV) inhibitors using the JFH-1 viral culture system and found that selective estrogen receptor modulators (SERMs), such as tamoxifen, clomifene, raloxifene, and other estrogen receptor α (ERα) antagonists, inhibited HCV infection. Treatment with SERMs for the first 2 h and treatment 2–24 h after viral inoculation reduced the production of HCV RNA. Treating persistently JFH-1 infected cells with SERMs resulted in a preferential inhibition of extracellular HCV RNA compared to intracellular HCV RNA. When we treated two subgenomic replicon cells, which harbor HCV genome genotype 2a (JFH-1) or genotype 1b, SERMs reduced HCV genome copies and viral protein NS5A. SERMs inhibited the entry of HCV pseudo-particle (HCVpp) genotypes 1a, 1b, 2a, 2b and 4 but did not inhibit vesicular stomatitis virus (VSV) entry. Further experiment using HCVpp indicated that tamoxifen affected both viral binding to cell and post-binding events including endocytosis. Taken together, SERMs seemed to target multiple steps of HCV viral life cycle: attachment, entry, replication, and post replication events. SERMs may be potential candidates for the treatment of HCV infection.

Keywords: HCV; Tamoxifen; SERM (Selective estrogen receptor modulator)

1. Introduction

Over 170 million people in the world are infected with the hepatitis C virus (HCV). Approximately 20% of infected patients develop cirrhosis and hepatocellular carcinoma after chronic HCV infection. No HCV vaccine is available yet, and the current standard of care, which consists of a combination of interferon (IFN) and ribavirin, is only effective for approximately 50% of infected patients, and many patients have serious side effects. Because of the urgent need for novel HCV therapeutics, research is being conducted to develop new anti-HCV drugs. In addition to *in vitro* screening assays that target HCV-specific enzymes, other approaches that use replicon cells and the recently described Huh 7.5.1-JFH-1 (genotype 2a)-infection system have been developed [1]. The Huh 7.5.1-JFH-1-infection system is an excellent system to identify HCV inhibitors that interfere with individual steps of the HCV life cycle, such as viral attachment, entry, and release. This experimental system allows both viral and host components that are involved in HCV infection to be targeted. Although drugs that target the host components may be toxic, such drugs are unlikely to select for resistant viruses.

We screened chemicals using a cell-based screening system [2] and found that tamoxifen and other selective estrogen receptor modulators (SERMs) inhibited HCV infection. Tamoxifen has been successfully used for the treatment of breast cancer since it was found to be an ER antagonist over 30 years ago. Clomifene and raloxifene, which are compounds

* Corresponding author. Tel.: +81 3 5285 1111x2327; fax: +81 3 5285 1272.
E-mail address: murakami@nih.go.jp (Y. Murakami).
[1] Present address: Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan.

1286-4579/$ - see front matter © 2012 Institut Pasteur. Published by Elsevier Masson SAS. All rights reserved.
http://dx.doi.org/10.1016/j.micinf.2012.10.003

that are related to tamoxifen, have been developed and used for the treatment of breast cancer and for the treatment of anovulation and osteoporosis. Currently, these three SERMs and toremifene have been approved in Japan and the US, and next-generation SERMs are undergoing clinical evaluation.

Because tamoxifen exhibited the ability to inhibit HCV infection, we determined which SERMs could effectively inhibit HCV infection and be approved for clinical use. The first-generation SERMs—tamoxifen, clomifene, and raloxifene—were all effective against HCV as were other ERα antagonists. We examined whether SERMs could be utilized as new drugs for the treatment of HCV.

2. Materials and methods

2.1. Cells and virus

Human hepatoma cell line, Huh 7.5.1 cells and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich Co. St. Louis, MO, USA) with 10% fetal bovine serum (FBS). HCV-JFH-1 (HCVcc) (genotype 2a) was the culture supernatant of infected Huh 7.5.1 cells as described previously [2]. A subgenomic replicon cell line, clone #4-1, which harbors the genotype 2a (JFH-1) [3,4], and clone #5-15, that harbors the genotype 1b HCV genome [5], were also cultured in DMEM with FBS.

2.2. Chemicals

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs, supported by a Grant-in-Aid for Scientific Research on the Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology of Japan. Tamoxifen, diethylstilbestrol, triphenylethylene, 17β-estradiol, and brefeldin A were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). Clomifene was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA), and hydroxytamoxifen ((*z*)-4-hydroxytamoxifen) and raloxifene were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Chloroquine was purchased from WAKO (Osaka, Japan). Other chemicals were purchased from Tocris Bioscience (Bristol, UK).

2.3. Quantification of the viral titer in medium

Huh 7.5.1 cells were seeded in 96-well plates at a density of 2×10^4 cells per well in a volume of 120 μl. The next day, 15 μl of media that contained the test compound and 15 μl of the HCVcc virus stock solution at a moi of 0.01 were added to each well. After 5 days, 100 μl of the culture supernatant was taken from each well, and viral RNA was extracted. Total RNA was also extracted from the cells. Quantitative real-time RT-PCR was then performed with One step SYBR PrimeScript RT-PCR Kit (Takara-Bio Co., Otsu, Japan) as described previously [2]. In the case of #4-1 replicon cell, as an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were measured with primers 5′-CCACCCATGGCAAATTCC-3′ and 5′-TGGGATTTCCATTGAT-3′. Cell growth was monitored using the MTT assay as described previously [6].

2.4. Western blotting

Western blotting was performed as previously described [2]. Briefly, cell lysates that contained equal quantities of protein were separated by SDS-PAGE, transferred onto PVDF membranes, and probed with antibodies against the core antigen (2H9), NS5A (Austral Biologicals, San Raman, CA, USA), or GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, USA). After incubation with horseradish peroxidase-conjugated secondary antibodies, the protein bands on the PVDF membranes were detected using an ECL system (GE Healthcare UK Ltd., Amersham Place, UK).

2.5. Production of and infection with pseudo-particles

HCV pseudo-particles (HCVpp) were generated using the following 3 plasmids: a Gag-Pol packaging construct (Gag-Pol 5349), a transfer vector construct (Luc 126), and a glycoprotein-expressing construct (HCV E1E2) (JFH-1, 2a). The generation of the pseudo-particles was performed according to the method described by Bartosch et al. [7]. To express the glycoproteins of other HCV genotypes, HCV E1E2 constructs of the genotypes 1a (H77), 1b (UKN1B 12.6), 2b (UKN2B 2.8), and 4 (UKN4 11.1) were generously provided by Dr. F. Cosset (INSERM, France) [8]. To produce VSVpp, a plasmid that coded the vesicular stomatitis virus (VSV) envelope, pCAG-VSV, was generously provided by Dr. Y. Matsuura (Osaka University, Japan). Gag-Pol 5349 (3.1 μg), Luc 126 (3.1 μg), and each of the individual glycoprotein-expression constructs (1.0 μg) were co-transfected into 293T cells that were seeded on a 10-cm dish (2.5×10^6 cells) using TransIT-LT1 Transfection Reagent (21.6 μl) (Mirus Bio LLC, Madison, WI, USA). The medium from the transfected cell cultures was harvested and used as the pseudo-particle stock. For the infection assay, Huh 7.5.1 cells were seeded onto a 48-well plate at a density of 4×10^4 cells per well one day prior to infection. The medium was then removed, and the cells were subsequently infected with the pseudo-particles in the presence or absence of drug. The cells were then incubated for 3 h. The VSVpp preparation was diluted (1:600) to infect with similar RLU activity compared to the HCVpp. The supernatant was then removed, fresh culture medium was added to the cells, and the cells were incubated for an additional 3 days. The luciferase assays were performed using a luciferase assay system (Promega Co. Madison WI, USA). Anti-CD81 antibody (sc-23962) was purchased from Santa Cruz Biotech.

3. Results

3.1. Tamoxifen and estrogen receptor α antagonists inhibited HCV infection

Using quantitative RT-PCR, we screened the compounds in the SCADS inhibitor kit I. Drugs and HCVcc at a moi of 0.01

were added to Huh 7.5.1 cells. Five days later, the quantity of HCV RNA in the culture supernatant was measured using quantitative real-time RT-PCR [2]. We found that tamoxifen reduced the levels of JFH-1 RNA in the culture supernatant. We also examined the effects of other SERMs and agonists and antagonists of ERα. As shown in Fig. 1, tamoxifen, clomifene, and hydroxytamoxifen, which have a triphenylethylene backbone, exhibited intense inhibitory effects (EC_{50}: approximately 0.1 μM). Triphenylethylene showed reduced inhibitory activity (data not shown). Raloxifene also inhibited viral RNA production at a similar concentration. (EC_{50}: approximately 0.1 μM) (Fig. 1a). Tamoxifen and raloxifene display both ERα antagonist and agonist properties in a dose- and tissue-dependent manner [9]. In contrast, ICI 182,780 (fulvestrant), ZK164015, and MPP (methyl-piperidino-pyrazole) are exclusively antagonistic [10–12]. These ERα antagonists also showed inhibitory activity against JFH-1, but their EC_{50} values were approximately 1 μM (Fig. 1b). As the 50% toxic concentrations (TC_{50}) for these compounds were observed to be greater than 10 μM (Fig. 1a and b), these specific indexes are over 100. In contrast, the ERα agonists 17β-estradiol, diethylstilbestrol, and PPT (1,3,5-tris(4-hydroxyphenyl)-4-propyl-1*H*-pyrozole) did not inhibit HCV (Fig. 1c). As expected, the SERMs that were observed to effectively inhibit HCV RNA production also reduced the core protein levels intracellularly (Fig. 1d).

3.2. SERMs inhibited more than one step of the JFH-1 life cycle

To determine which step of the JFH-1 life cycle was inhibited by the SERMs studied, we performed time-of-addition experiments. As described previously [2], JFH-1 appears to complete one infectious life cycle in approximately 48 h. Huh 7.5.1 cells were inoculated with JFH-1-containing medium (moi 0.1) with or without drug and were then incubated for 2 h. After the medium was removed, fresh medium with or without drug was added. The cells were then incubated for another 46 h. Treatment with 10 μM tamoxifen for 48 h reduced the amount of viral RNA in the medium to 1.7% of levels observed in the control. Treatment with tamoxifen for the first 2 h after infection (0–2 h) reduced viral RNA to 2.3% of the levels observed in the control. The addition of tamoxifen to the fresh medium just after the removal of the virus (2–48 h) resulted in a reduction in the amount of viral RNA to 10.7% of the levels observed in the control. The addition of tamoxifen 24 h after viral inoculation (24–48 h) resulted in a decrease in the amount of viral RNA to 60% of the levels observed in the control (Fig. 2a). This result suggests that tamoxifen inhibits mainly viral entry and some steps during replication. 10 μM of raloxifene exhibited a similar inhibitory pattern but less inhibited by the treatment after the entry step (Fig. 2b). A pure ERα antagonist, ICI 182,780 (30 μM), also exhibited inhibition of both viral entry and the replication steps, but the inhibition of the entry step was not so marked (Fig. 2c).

To further investigate effect on HCV post replication, we infected HCV in the presence of the drugs for 72 h (moi 0.1) and examined their effects on intracellular and extracellular HCV RNA levels. Brefeldin A, an inhibitor of protein transport [13], was used as a positive control of post replication inhibition. In this experimental setting, brefeldin A showed intracellular HCV RNA accumulation suggesting post replication inhibition (Fig. 2d). SERMs generally reduced HCV RNA in cell as well as HCV RNA in medium, although the extent of reduction was different (Fig. 2d). Lower concentration of SERMs reduced extracellular HCV RNA more robustly than intracellular HCV RNA. At a concentration of 0.1 μM, tamoxifen exclusively inhibited HCV RNA in the culture supernatant but not intracellular HCV RNA levels, in a manner similar to that of brefeldin A (Fig. 2d). The results suggest that SERMs inhibit post replication step(s) such as assembly or release. Because low concentrations of tamoxifen failed to inhibit intracellular HCV RNA, SERMs potentially target post replication step(s) more efficiently than replication step. In this condition, higher concentrations (1 and 3 μM) of tamoxifen seemed to inhibit intracellular HCV RNA rather than extracellular HCV RNA, although the reason is not clear.

To determine the effect of these drugs on chronic infection, we used pre-infected Huh 7.5.1 cells. We infected the cell with HCVcc at a moi of 0.01 and incubated for 3 days. Three days after infection, the drugs were added, and the cells were further incubated for 48 h. At the time of drug addition, the cells were persistently infected, and HCVcc was continuously produced and released into the culture supernatant, which is similar condition to chronic infection. HCV RNA was extracted from the culture supernatant and the cells after 48 h and measured copy number of HCV RNA. Both HCV RNA in the culture supernatant and that in the cell were reduced by treatment with the SERMs, but the intracellular HCV RNA levels were less reduced (Fig. 2e). This suggested that the SERMs caused preferential reduction in extracellular HCV RNA through interference with some post replication step(s), such as assembly or release. Brefeldin A accumulated intracellular HCV RNA, and reduced HCV RNA level in the culture supernatant (Fig. 2e).

These data suggested that the SERMs inhibit multiple steps in the HCV life cycle: entry, viral RNA replication and some post replication step(s).

3.3. SERMs inhibited copies and NS5A protein expression in replicon cells

To confirm the effect of these drugs on viral replication, we used two subgenomic replicon cells. The subgenomic replicon cells, derived from Huh7 cells, harbor HCV viral RNA that replicates autonomously, and they express viral proteins. We treated cells that harbored a subgenomic replicon (#4-1, genotype 2a) [3,4] with the SERMs for 48 h and measured the amount of cellular replicon RNA by quantitative RT-PCR. Treatment with 10 μM of tamoxifen, raloxifene, or 3 μM of clomifene, inhibited HCV RNA compare to GAPDH RNA, although statistical significance was shown in only the inhibition of 10 μM of tamoxifen. ICI 182,780 did not show specific inhibition of HCV RNA (Fig. 3a).

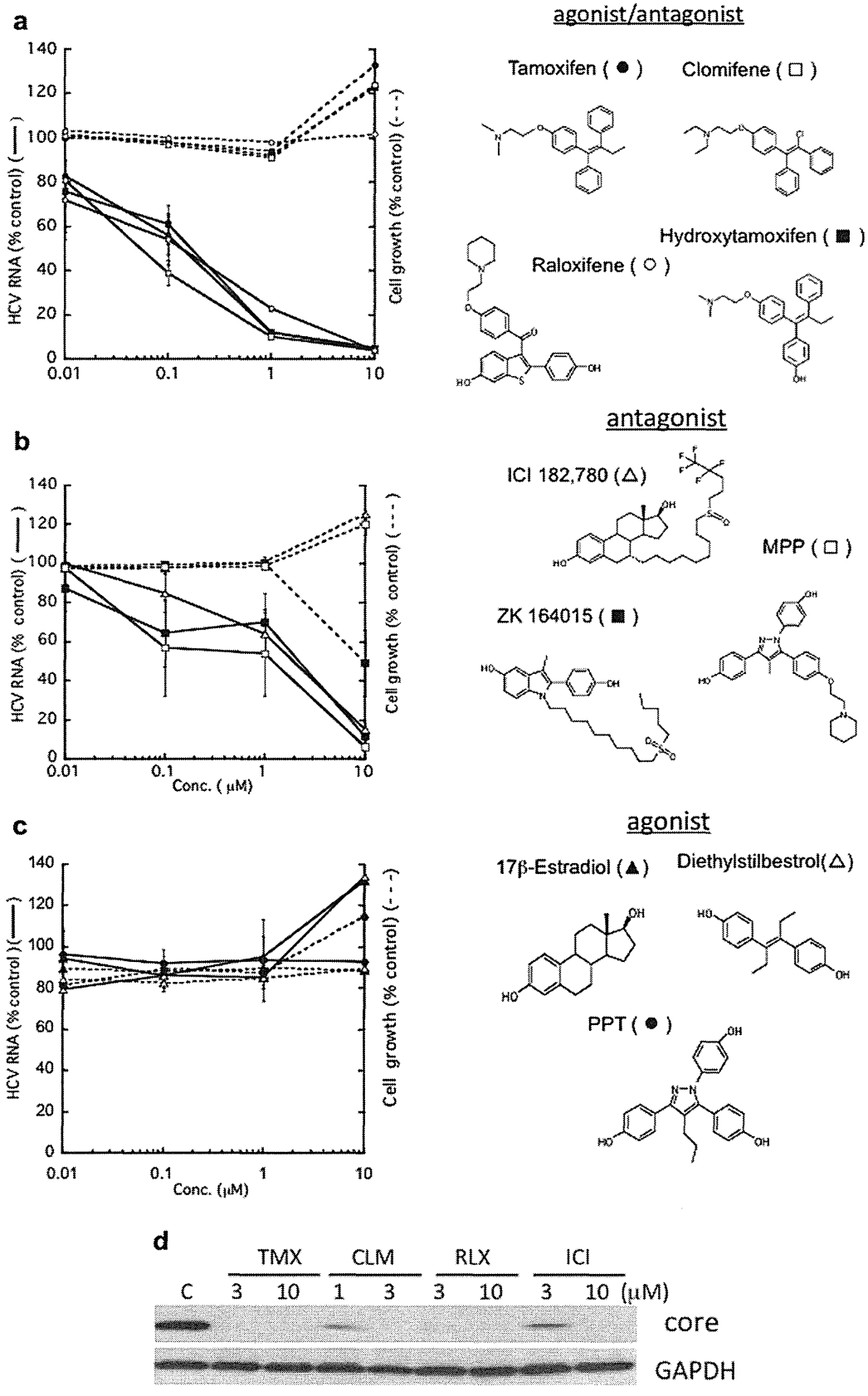


Fig. 1. Effects of SERMs on JFH-1 HCV RNA levels. a) Effects of tamoxifen, clomifene, and raloxifene. Huh 7.5.1 cells were infected with HCV JFH-1 (moi 0.01) in the presence of drugs and were incubated for 5 days. Drugs were added just before viral inoculation. HCV RNA in the medium was measured by tube-capture-RT-PCR [2]. Parallel cultures of cells without virus were analyzed using the MTT assay to detect the inhibition of cell growth due to drug exposure. Tamoxifen (closed circles), clomifene (open rectangles), hydroxytamoxifen (closed rectangles), and raloxifene (open circles). The percentages to control HCV RNA and

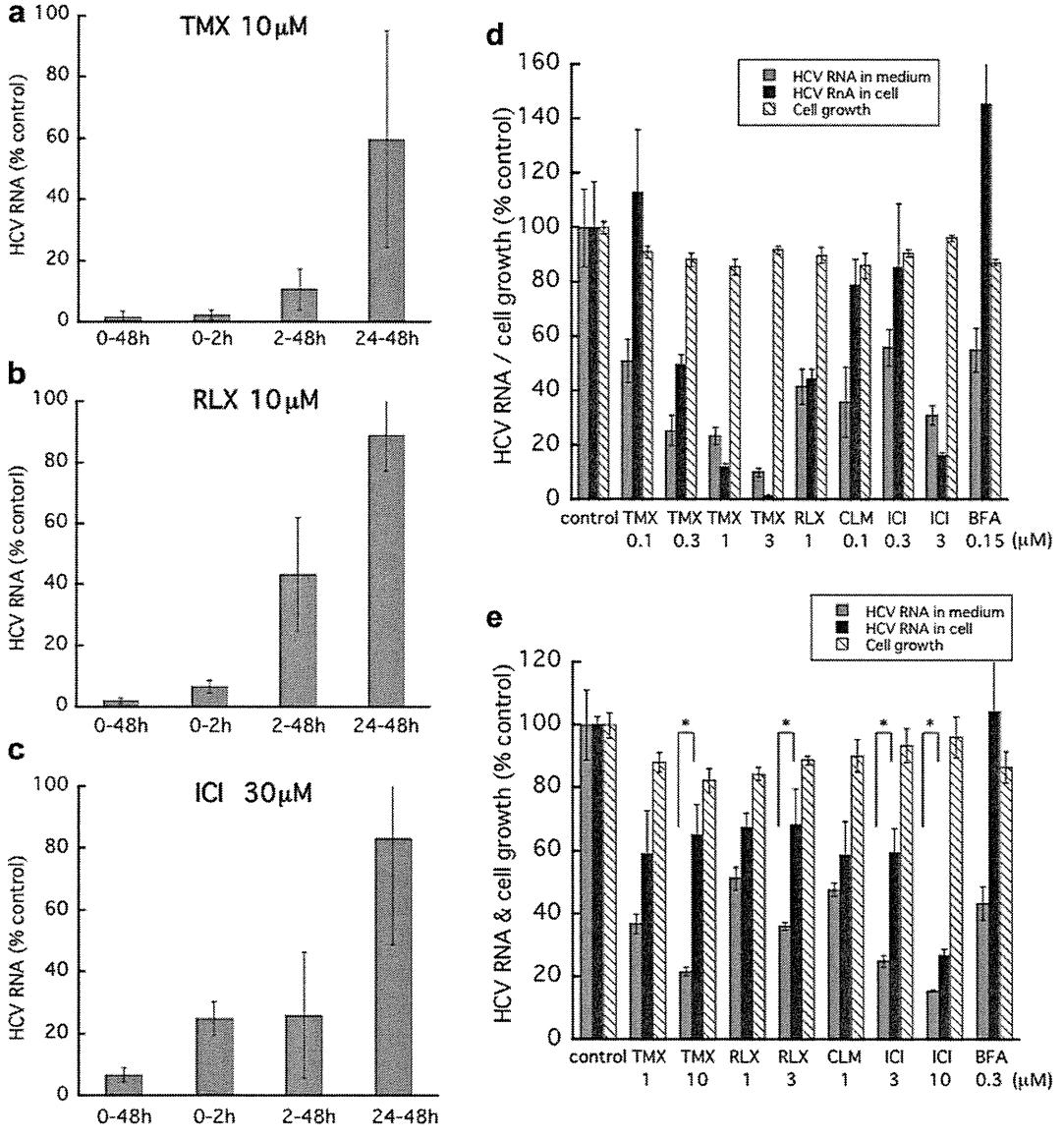


Fig. 2. Time-of-addition experiments (a–c) and the effect of SERMs on extracellular and intracellular HCV RNA in simultaneous infection (d) and in persistent infection (e). Huh 7.5.1 cells were treated with tamoxifen (TMX) (10 μM, a), raloxifene (RLX) (10 μM, b), or ICI 182,780 (ICI) (30 μM, c) during the following time periods: 0–2 h, 2–48 h, or 24–48 h after JFH-1 infection (moi 0.1). Forty-eight hours after infection, the culture supernatant was harvested, and HCV RNA was extracted and subjected to quantitative real-time RT-PCR to determine the number of copies of the JFH-1 genome. The data are the averages of three independent experiments and the standard deviation. d) Effect of treatment with SERMs for 3 days on extra- and intra-cellular HCV RNA levels. Huh 7.5.1 cells were infected with JFH-1 (moi 0.1) just after addition of the SERMs. Three days later, RNA was extracted from the cells and from the culture supernatant. The amount of HCV RNA was measured by quantitative real-time RT-PCR. Brefeldin A (BFA) was used as a positive control. e) Huh 7.5.1 cells were infected at a moi of 0.01, 3 days before addition of drugs. The infected cells were treated with SERMs for 48 h. RNA was subsequently extracted from the cells and the culture supernatant to determine the viral genome copy number. The results are presented as the percentage of control cells without drug. The data are the averages of triplicates and the error bars represent standard deviation. *P-value < 0.05. One representative experiment of two independent experiments is shown.

Next we treated this (#4-1, genotype 2a) and another replicon (#5-15, genotype 1b) [5] with the SERMs for 3 days and examined the effect of the compounds on the HCV NS5A protein levels by western blotting. As shown in Fig. 3b, the SERMs except ICI 182,780 reduced the level of NS5A in accordance with the results in Fig. 3a. ICI 182,780 seemed to slightly reduce NS5A protein in #5-15 replicon cell. The SERMs did not reduce the protein levels of GAPDH in the subgenomic replicon cells (Fig. 3b). These results indicated that SERMs, at least tamoxifen, raloxifene and clomifene,

control cell growth are indicated by solid lines and dotted lines, respectively. b) Effect of the following ERα antagonists: ICI 182,780 (closed triangles), ZK164015 (closed rectangles), and MPP (open rectangles). c) Effect of the following ERα agonists: 17β-estradiol (closed triangles), diethylstilbestrol (open rectangles), and PPT (closed circles). The results are presented as percentages of the control cells that were not treated with drugs. Values are the averages of triplicates, and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown. d) Huh 7.5.1 cells were infected (moi 0.01) in the presence of tamoxifen (TMX), clomifene (CLM), raloxifene (RLX), or ICI 182,780 (ICI) and incubated for 5 days. Cell lysates were blotted with anti-core and anti-GAPDH antibodies as described in the Section Materials and methods.

were effective not only against HCV genotype 2a but also HCV genotype 1b and that the compounds inhibited a HCV replication step. The growth of the replicon cells was suppressed by treatment with 10 μM of clomifene. Clomifene at concentrations less than 10 μM and tamoxifen, raloxifene and ICI 182,780 at 10 μM concentration or lower did not inhibit cell growth (Fig. 3c).

3.4. SERMs inhibited entry of HCVpp but not VSVpp

To further examine the inhibition of early viral processes by the SERMs, we used infectious HCV pseudo-particles (HCVpp). Because HCVpp enter into cell dependent on HCV envelope protein but replicate dependent on retroviral system in the cell, we can exclude other effects of the drug except effect on HCV entry system. Pseudo-particles with the viral envelope glycoprotein mimic the entry of the parental virus, and this system has been used for investigation of HCV entry [7,8,18,20,21]. The infectious titer is determined by luciferase activity. We added tamoxifen to HCVpp- or VSVpp-containing medium and incubated Huh 7.5.1 cells with this medium for 3 h. After washing the cells, fresh medium was added, and the cells were incubated for 3 days. Treatment with tamoxifen reduced the luciferase activity of the cells that were infected with HCVpp in a dose-dependent fashion. In contrast, the luciferase activity caused by VSVpp was not reduced by the same concentrations of tamoxifen (Fig. 4a). We also examined the effect of other SERMs, such as clomifene, raloxifene, ICI 182,780, ZK164015, and MPP, on HCVpp infection. All of these SERMs inhibited the luciferase activity caused by HCVpp but not the activity caused by VSVpp (Fig. 4b). ICI 182,780 showed a weaker effect compared to tamoxifen, clomifene and raloxifene. Next, we examined the effects of these drugs on various genotypes of HCVpp. Although the extent of inhibition was varied, the compounds inhibited all of the genotypes that were examined (Fig. 4c). At a concentration of 10 μM, ICI 182,780 inhibited all of the genotypes of HCVpp other than genotype 2a. These results suggested that the SERMs inhibit entry of all genotypes of HCV.

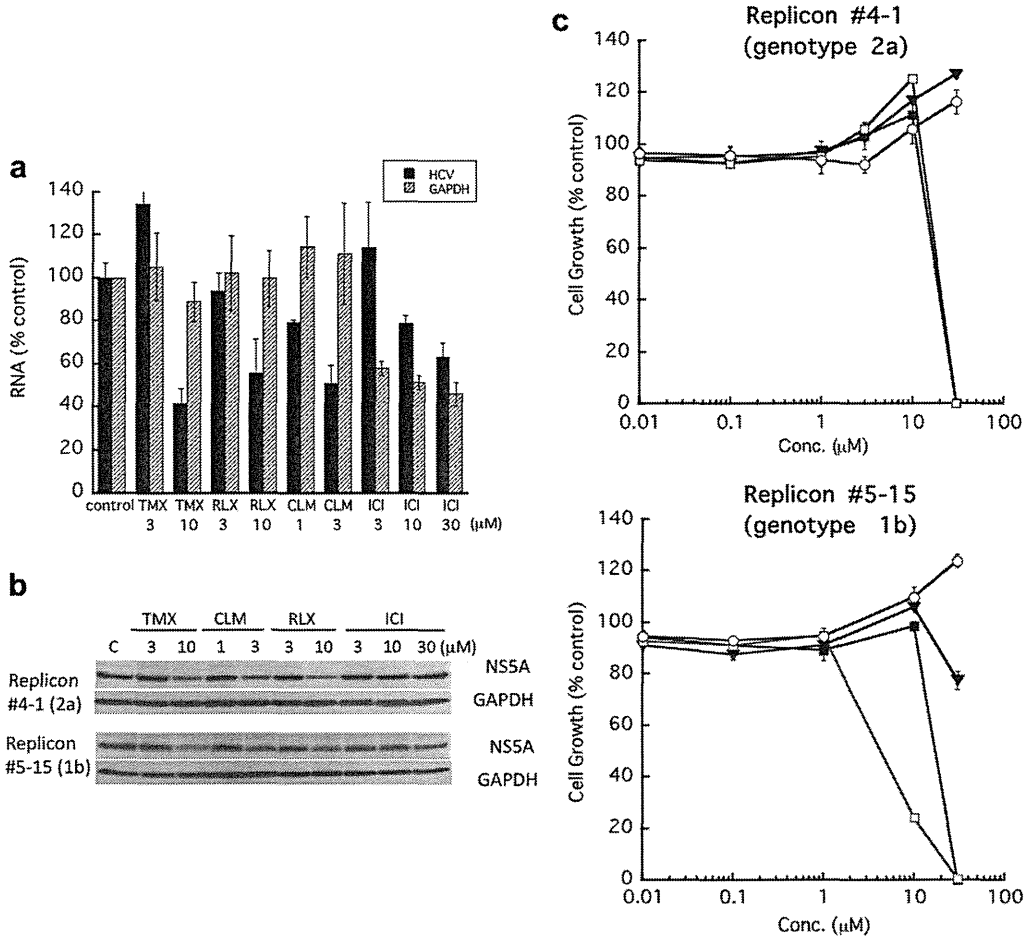


Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount of GAPDH RNA was measured and indicated as percentage of control cells without drug (a). Another subgenomic-replicon-harboring cell line, clone #5-15 (genotype 1b) was treated with SERMs for 3 days. Cell lysates were subjected to western blotting with an anti-NS5A antibody or an anti-GAPDH antibody (b). Cells that were grown for 3 days in the presence of tamoxifen (closed rectangles), clomifene (open rectangles), raloxifene (closed triangles), or ICI 182,780 (open circles) were measured using the MTT assay. Cell growth is expressed as a percentage of control cells without drug (c). The values are the average of triplicate and the error bars represent the standard deviation of the mean. One representative experiment of two independent experiments is shown.

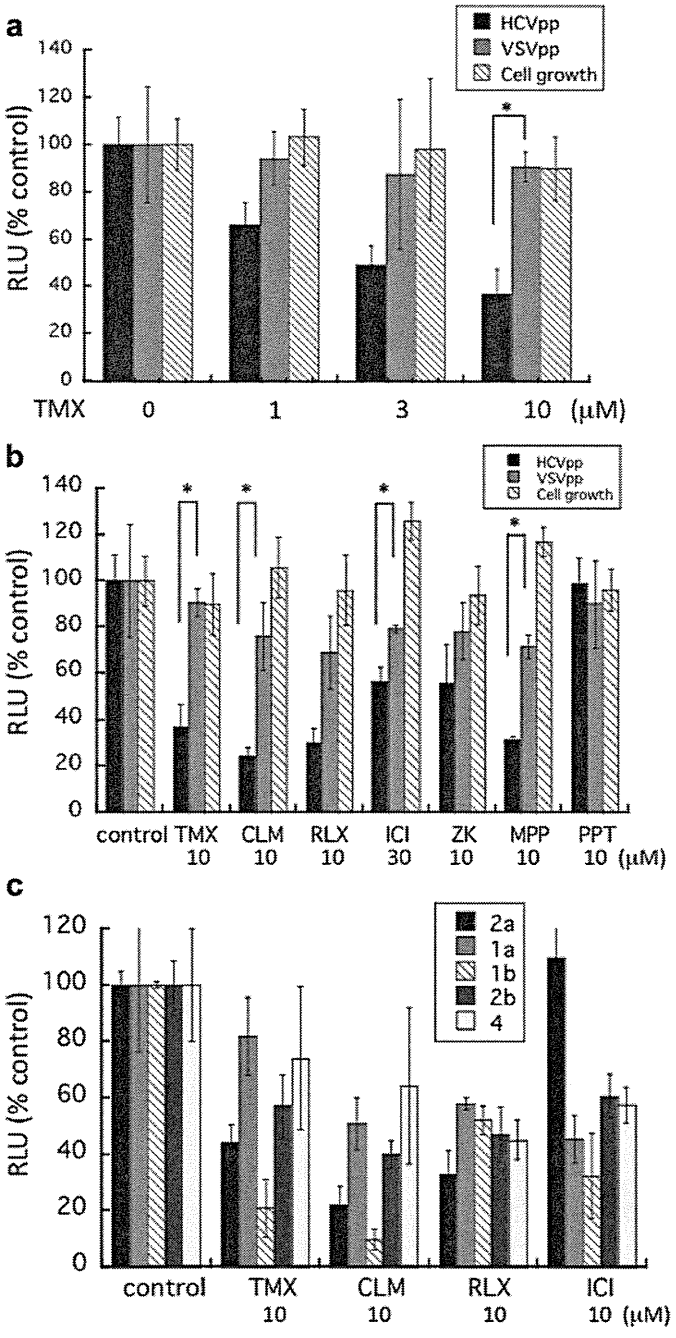


Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 3 days. The VSVpp preparation was diluted 600 times so it was infected at similar RLU activity levels compared to HCVpp (approximately 5000 RLU). A parallel culture without pseudo-particles was analyzed using the MTT assay to evaluate the effect of the drugs on cell growth. b) Effects of various SERMs on HCVpp and VSVpp infection. c) Effects of SERMs on the various genotypes of HCVpp infection. The control luciferase activities were approximately 5000 RLU (genotype 2a), 3000 RLU (genotype 1a), 2400 RLU (genotype 1b), 3900 RLU (genotype 2b), and 860 RLU (genotype 4). The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars are the standard deviation of the mean. *P-value < 0.05. One representative experiment of three independent experiments is shown.

3.5. Effect of tamoxifen on the attachment and entry steps

To better understand how tamoxifen blocks HCV entry, we performed an experiment to discriminate between the inhibition of HCV attachment to cells and the inhibition of post-binding entry events. HCV attaches to several cellular receptors via its E1 and E2 envelope proteins and enters via clathrin-mediated endocytosis [14–16]. We used HCVpp because infection with HCVpp is thought to simulate HCV entry [7,17,18] and the entry is independent of HCV replication. HCVpp binding to the cellular receptors was performed at 4 °C for 1.5 h. Under these conditions, HCVpp bind to the cells but entry is not efficient. The inoculum was removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. In protocol I, the drug was administered during the binding step at 4 °C. After the shift to 37 °C, treatment with the drug was performed during first hour (protocol II) or after 1 h at 37 °C (protocol III) to distinguish between the inhibition of early and late post-binding events (Fig. 5a). The inoculum was removed after treatment, and fresh medium was added to the cells. We used chloroquine, a lysosome-tropic agent, as a control inhibitor for early entry (protocol II) [19]. We also used an anti-CD81 antibody that specifically inhibits HCV entry through the inhibition of the HCV cellular receptor protein CD81 at early entry [20,21]. As expected, chloroquine inhibited luciferase activity when the cells were treated during the early post-binding step (protocol II). This result suggested that endocytosis occurred primarily during the first post-binding period (protocol II). Anti-CD81 markedly inhibited luciferase activity during protocol II as reported [18,19]. Tamoxifen treatment did not result in clear differences between the protocols and the compound displayed similar activity regardless of the treatment period (Fig. 5b left). As a control, the same experiment was performed using VSVpp. Chloroquine inhibited the early entry step of VSVpp, but anti-CD81 and tamoxifen did not show any inhibition (Fig. 5b right).

Tamoxifen is a lipophilic weak base and inhibits acidification intracellularly [22]. Therefore, we examined whether the inhibition of the endocytosis of HCVpp by tamoxifen was dependent on its function as a weak base. Chloroquine is a weak base and inhibits endosome acidification. The pH sensitivity is considered a good indication of clathrin-dependent endocytosis. Previous reports have indicated that chloroquine inhibited HCVcc and HCVpp infection [14,19]. We adjusted the medium to pH 5.5 and incubated the cells in this acidic medium in the presence or absence of tamoxifen for 2 h post-binding. The acidification of the medium did not affect either the entry of HCVpp or the cell growth (Fig. 5c). Treatment with tamoxifen in the medium with a normal pH (pH 7.1) reduced HCVpp entry, and treatment with the drug in the acidic medium also reduced entry to a similar extent. In contrast, chloroquine treatment in regular medium reduced HCVpp entry, but entry was restored in the acidic medium (Fig. 5c). These results indicate that the inhibitory effect of tamoxifen was not dependent on the function of this compound as a base, unlike the effects of chloroquine.

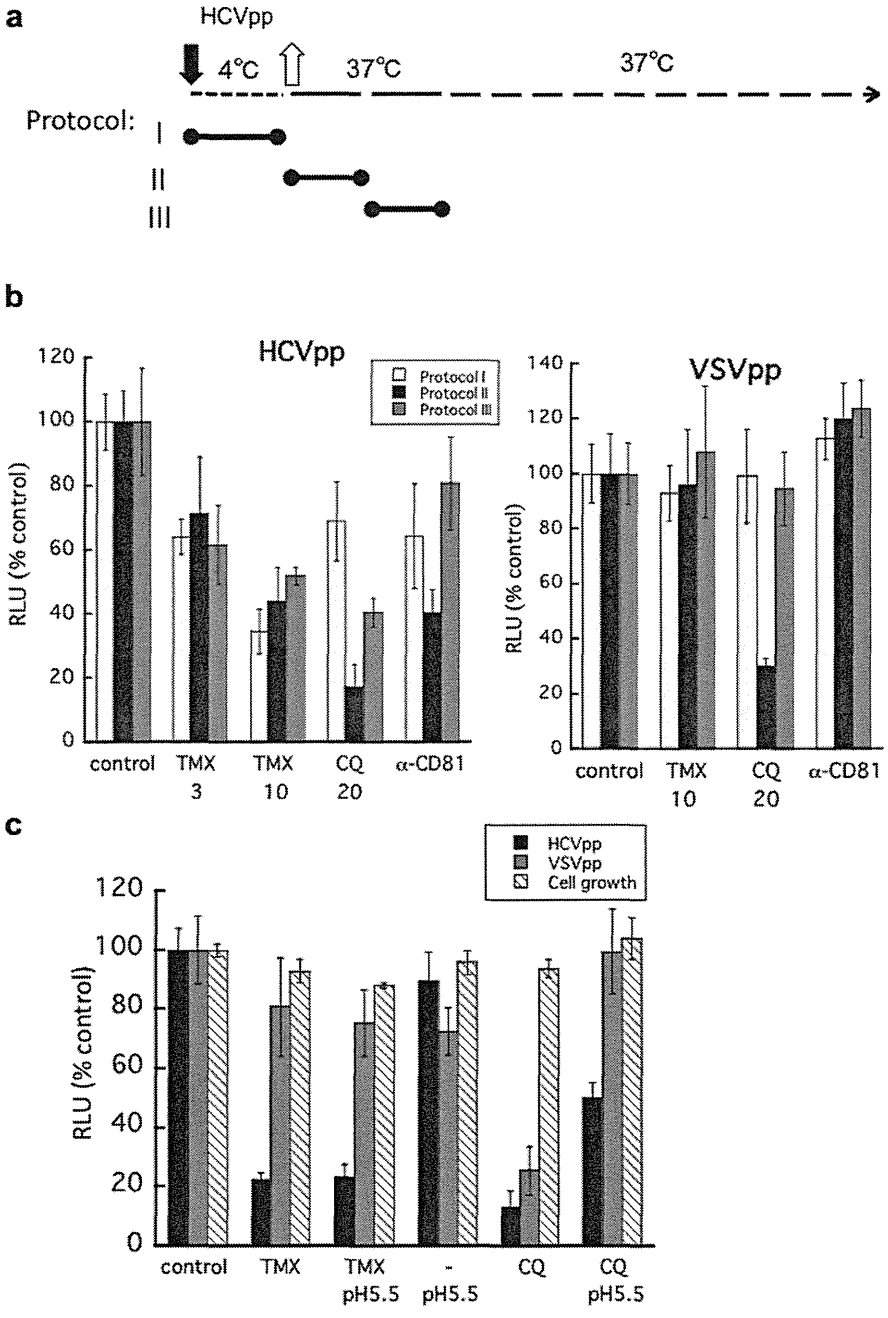


Fig. 5. Effect of tamoxifen on the attachment and endocytosis of HCVpp. a) Experimental design. HCVpp attachment to cells was performed at 4 °C for 1.5 h in the presence or absence of drug. Under these conditions, HCVpp bind to the cells but do not efficiently enter the cells. The inoculum was then removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. The cells were treated with drug during the binding period at 4 °C (protocol I), during first hour after the shift to 37 °C (protocol II), or 1 h after the shift to 37 °C (protocol III). The drug-containing medium was removed for every treatment, and new medium was supplied to the cells. b) Effects of tamoxifen (TMX) (3 and 10 μM), chloroquine (CQ) (20 μM) and anti-CD81 antibody (20 μg/ml) on HCVpp attachment (protocol I) and post-binding events (protocol II and III) (left). Effects of tamoxifen, chloroquine and anti-CD81 on VSVpp (right). c) Effects of exposure to low pH on the inhibition of HCVpp entry by tamoxifen and chloroquine. The cells were incubated with HCVpp at 4 °C for 1.5 h in the absence of drug. After removing the inoculum, regular (pH 7.1) or acidic medium that was adjusted with HCl to pH 5.5, either with or without drug (tamoxifen, 10 μM, chloroquine, 20 μM), was added to the cells. The cells were subsequently incubated at 37 °C. The drug-containing medium was removed after 2 h of incubation, and the cells were incubated for an additional 3 days with fresh, regular medium. The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown.

4. Discussion

We screened for HCV inhibitors using the JFH-1-Huh 7.5.1 cell culture system and found that tamoxifen and ERα antagonists, but not ERα agonists, inhibited HCV JFH-1 infection. Although there are some reports about the HCV inhibitory effects of tamoxifen and other SERMs, we presented further information about the inhibitory effects of these substances. The time-of-addition experiments (Fig. 2a–c) suggested that these SERMs inhibit the entry and replication

steps in the HCV life cycle. These SERMs, except ICI 182,780, reduced level of HCV genome (genotype 2a) and NS5A (genotypes 1b and 2a) in the subgenomic replicon cells (Fig. 3), which supports the hypothesis that the inhibitory effect of the SERMs occurred during the replication steps. Further we observed that SERMs preferentially reduced extracellular HCV RNA compare to intracellular HCV RNA in the newly (Fig. 2d) and persistently infected cells (Fig. 2e). It suggests that the SERMs also target post replication step(s) in the viral life cycle, such as assembly and release. A low concentration of tamoxifen (0.1 μM) accumulated intracellular HCV RNA (Fig. 2d), which suggests that SERMs target post replication step(s) more efficiently than replication steps. Additionally, these compounds inhibited HCVpp infection (Fig. 4), which supports an inhibitory effect during the entry step. The inhibition of entry was mediated through the inhibition of viral binding to cells and through the inhibition of a post-binding event (Fig. 5b). Taken together, SERMs seemed to target multiple steps of the HCV viral life cycle.

Among the SERMs, ICI 182,780 did not exhibit distinct inhibition of replication in the replicon cells (Fig. 3a and b), although the compound seemed to inhibit entry and replication steps according to the result of time-of-addition experiment (Fig. 2c). The replicon cells are derived from Huh 7 cell. Although viral sensitivity to the SERMs might be dependent on the cell that HCV infect, it remains unclear whether ICI 182,780 inhibits replication step or not. However, the compound affected post replication step in a similar manner to other SERMs (Fig. 2d and e). It is unlikely that ICI 182,780 is an inhibitor with different mechanisms.

The inhibitory effect of tamoxifen on HCV replication has been previously reported by Watashi et al. [23]. They also demonstrated that ICI 182,780 inhibited HCV replication. This effect was discovered using a cell line that harbored a subgenomic replicon (genotype 1b). Additionally, these researchers reported that RNA interference-mediated knockdown of ERα, not ERβ, reduced HCV replication, but the reduction was not related to ERE-mediated transactivation activity. They suggested that ERα interacts with the HCV viral polymerase NS5B and that ERα promotes the participation of NS5B with the HCV replication complex. Using the Huh 7.5.1-JFH-1 screening system, Gastaminza et al. and Chockalingam et al. found that toremifene and raloxifene, respectively, function as HCV inhibitors. Gastaminza et al. [24] reported that toremifene inhibited HCV infection by inhibiting both the entry and release steps of the viral life cycle. Chockalingam et al. [25] determined that raloxifene inhibited the entry and replication steps, as we also observed. Our results are in accordance with these previous reports and other information about the inhibitory effects of SERMs.

Pseudo-particle experiments confirmed that SERMs affected the entry step of HCV viral life cycle (Fig. 4b), although the inhibitory effects were lower compare to those against HCVcc (Fig. 2a–c). The reason for the difference in sensitivity may account for some difference in the two entry systems. Otherwise, in the treatment with the drugs for the first 2 h of HCVcc infection, some amount of the drugs might enter the cell and remain affecting the other steps. The SERMs affected not only genotype 2a but also other genotypes of HCVpp that were examined, suggests that these chemicals have effects on various genotypes of HCV. Although the SERMs appeared to inhibit multiple steps of the HCV life cycle, the primary target step in the viral life cycle might be the entry step. In the time-of-addition experiments, treatment with tamoxifen or raloxifene during the first 2 h was more effective than treatment during the subsequent 2–48 h (Fig. 2a). These SERMs are thought to primarily prevent viral entry and to inhibit post replication step and replication at higher concentrations.

As shown in Fig. 5, an experiment that could discriminate an effect on viral attachment from an effect at the post-binding processes indicated that tamoxifen inhibited both steps. The inhibition of endocytosis by tamoxifen was not rescued by exposure to a low pH. This suggests that the observed inhibition is the result of a mechanism that is independent of the compound's function as a base. HCV entry is a highly complicated process that involves numerous viral and cellular factors. Tamoxifen is thought to target multiple steps that are involved in the attachment and entry steps of the HCV life cycle, which results in high levels of inhibition.

At present, the mechanism of the entry inhibition by SERMs is not clear. It is possible that tamoxifen targets viral molecules, but we have no evidence to support this hypothesis. ERα might be a target molecule because all of the antagonists of ERα that were examined had an inhibitory effect. Watashi et al. indicated that ERα is involved in HCV replication [23]. ERα is thought to be present in the cytoplasm, which is where HCV replicates. However, it is doubtful that ERα is present on the cell surface where viral entry occurs. The addition of 17β-estradiol with tamoxifen did not prevent the inhibitory effect of tamoxifen in the HCVpp experiment (data not shown). This result suggests that tamoxifen does not compete with 17β-estradiol for the target molecules involved in HCV entry. Additionally, a pure ERα antagonist, ICI 182,780, was a less effective inhibitor of the entry step. Based on these results, it is thought that the molecule responsible for HCV entry that is targeted by SERMs is not ERα.

Tamoxifen has various targets other than ERα, such as P-glycoprotein (GPR30), calmodulin, and protein kinase C [26]. GPR30 (G protein-coupled receptor protein 30) is a membrane-associated estrogen receptor that is distinct from the classical ER [27]. Tamoxifen and ICI 182,780 are agonists of GPR30 [28]. We examined the effect of a specific GPR30 agonist, G-1, and a GPR30 antagonist, G-15, on HCVpp infection [29]. G-1 and G-15 did not inhibit HCVpp infection. Conversely, HCVpp infection was observed to increase upon addition of these compounds (data not shown). This result suggested that GPR30 is not involved in the inhibition of HCV entry.

We previously reported that a typical PKC inhibitor, bisindolylmaleimide I (BIM I), inhibited HCV replication [2]. BIM I (10 μM) inhibited both HCVpp and VSVpp infection in a similar manner by approximately 50% (data not shown). This suggests that BIM I has a different mechanism for the

inhibition of entry compared to tamoxifen. PKC is not thought to be involved in the HCV-specific inhibition of entry by SERMs.

There were few reports of HCV entry inhibitors until the development of the cell-culture JFH-1 infection system. It has recently been reported that fluphenazine, trifluoperazine and related chemicals exhibit a strong, dose-dependent inhibition of HCV entry without significantly affecting the entry of VSVpp [24,25]. These compounds are structurally similar to chlorpromazine, which is an inhibitor of the clathrin-coated pit formation that is required for HCV entry [14]. Interestingly, these compounds and the SERMs have a common structural characteristic: planar, multiple aromatic rings with a tertiary amine side chain. Tamoxifen, raloxifene and ERα antagonists all have this structure, but the ERα agonists do not have these structures. Fluphenazine and related chemicals may inhibit HCV entry through a mechanism that is similar to tamoxifen.

In summary, we observed a significant HCV inhibitory effect of various SERMs using the Huh 7.5.1 cell-JFH-1 infection system. Additionally, we demonstrated that SERMs could be useful for the treatment of HCV. Because it takes a great deal of time and money to develop a new drug from a novel chemical compound, it may be easier to use previously developed drugs that can be used for new applications. Tamoxifen, toremifene, and raloxifene are all drugs that have been in use for an extended period of time. In our present *in vitro* study, the effective concentrations for the HCV inhibitory effects of the SERMs were approximately 0.1–10 μM. In the case of tamoxifen, 20 mg per day, administered for 8 weeks resulted in plasma concentrations of approximately 0.5 μM. These concentrations could be sufficient to exert an anti-HCV effect. SERMs should be investigated to determine their efficacy for treating HCV clinically. Further examination of the mechanism of the entry inhibition mediated by SERMs would produce significant new data relevant to the understanding of HCV entry.

Acknowledgments

We thank Drs. Kyoko Murakami, Kenichi Morikawa, Tomoko Date, and Koichi Watashi for helpful advice. We also thank Drs. François-Loïc Cosset (INSERM, France) and Yoshiharu Matsuura (Osaka University, Japan) for generously providing plasmids. This study was supported by a grant-in-aid from the Ministry of Health, Labour and Welfare of Japan.

References

[1] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.

[2] Y. Murakami, K. Noguchi, S. Yamagoe, T. Suzuki, T. Wakita, H. Fukazawa, Identification of bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication by tube-capture-RT-PCR, Antivir. Res. 83 (2009) 112–117.

[3] T. Kato, T. Date, M. Miyamoto, A. Furusaka, K. Tokushige, M. Mizokami, T. Wakita, Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon, Gastroenterology 125 (2003) 1808–1817.

[4] T. Date, T. Kato, M. Miyamoto, Z. Zhao, K. Yasui, M. Mizokami, T. Wakita, Genotype 2a hepatitis C virus subgenomic replicon can replicate in HepG2 and IMY-N9 cells, J. Biol. Chem. 279 (2004) 22371–22376.

[5] V. Lohmann, F. Korner, J. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, Science 285 (1999) 110–113.

[6] H. Fukazawa, S. Mizuno, Y. Uehara, A microplate assay for quantitation of anchorage-independent growth of transformed cells, Anal. Biochem. 228 (1995) 83–90.

[7] B. Bartosch, J. Dubuisson, F.L. Cosset, Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes, J. Exp. Med. 197 (2003) 633–642.

[8] D. Lavillette, A.W. Tarr, C. Voisset, P. Donot, B. Bartosch, C. Bain, A.H. Patel, J. Dubuisson, J.K. Ball, F.L. Cosset, Characterization of host-range and cell entry properties of the major genotypes and subtypes of hepatitis C virus, Hepatology 41 (2005) 265–274.

[9] C.K. Osborne, H. Zhao, S.A. Fuqua, Selective estrogen receptor modulators: structure, function, and clinical use, J. Clin. Oncol. 18 (2000) 3172–3186.

[10] A.E. Wakeling, M. Dukes, J. Bowler, A potent specific pure antiestrogen with clinical potential, Cancer Res. 51 (1991) 3867–3873.

[11] C. Biberger, E. von Angerer, 2-Phenylindoles with sulfur containing side chains. Estrogen receptor affinity, antiestrogenic potency, and antitumor activity, J. Steroid Biochem. Mol. Biol. 58 (1996) 31–43.

[12] J. Sun, Y.R. Huang, W.R. Harrington, S. Sheng, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Antagonists selective for estrogen receptor alpha, Endocrinology 143 (2002) 941–947.

[13] N. Sciaky, J. Presley, C. Smith, K.J. Zaal, N. Cole, J.E. Moreira, M. Terasaki, E. Siggia, J. Lippincott-Schwartz, Golgi tubule traffic and the effects of brefeldin A visualized in living cells, J. Cell Biol. 139 (1997) 1137–1155.

[14] E. Blanchard, S. Belouzard, L. Goueslain, T. Wakita, J. Dubuisson, C. Wychowski, Y. Rouille, Hepatitis C virus entry depends on clathrin-mediated endocytosis, J. Virol. 80 (2006) 6964–6972.

[15] L. Meertens, C. Bertaux, T. Dragic, Hepatitis C virus entry requires a critical postinternalization step and delivery to early endosomes via clathrin-coated vesicles, J. Virol. 80 (2006) 11571–11578.

[16] D.M. Tscherne, C.T. Jones, M.J. Evans, B.D. Lindenbach, J.A. McKeating, C.M. Rice, Time- and temperature-dependent activation of hepatitis C virus for low-pH-triggered entry, J. Virol. 80 (2006) 1734–1741.

[17] A. Op De Beeck, C. Voisset, B. Bartosch, Y. Ciczora, L. Cocquerel, Z. Keck, S. Foung, F.L. Cosset, J. Dubuisson, Characterization of functional hepatitis C virus envelope glycoproteins, J. Virol. 78 (2004) 2994–3002.

[18] B. Bartosch, F.L. Cosset, Cell entry of hepatitis C virus, Virology 348 (2006) 1–12.

[19] E.G. Cormier, R.J. Durso, F. Tsamis, L. Boussemart, C. Manix, W.C. Olson, J.P. Gardner, T. Dragic, L-SIGN (CD209L) and DC-SIGN (CD209) mediate transinfection of liver cells by hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 14067–14072.

[20] E.G. Cormier, F. Tsamis, F. Kajumo, R.J. Durso, J.P. Gardner, T. Dragic, CD81 is an entry coreceptor for hepatitis C virus, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 7270–7274.

[21] G. Koutsoudakis, A. Kaul, E. Steinmann, S. Kallis, V. Lohmann, T. Pietschmann, R. Bartenschlager, Characterization of the early steps of hepatitis C virus infection by using luciferase reporter viruses, J. Virol. 80 (2006) 5308–5320.

[22] N. Altan, Y. Chen, M. Schindler, S.M. Simon, Tamoxifen inhibits acidification in cells independent of the estrogen receptor, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 4432–4437.

[23] K. Watashi, D. Inoue, M. Hijikata, K. Goto, H.H. Aly, K. Shimotohno, Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with viral RNA polymerase NS5B, J. Biol. Chem. 282 (2007) 32765–32772.

[24] P. Gastaminza, C. Whitten-Bauer, F.V. Chisari, Unbiased probing of the entire hepatitis C virus life cycle identifies clinical compounds that target multiple aspects of the infection, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 291–296.

[25] K. Chockalingam, R.L. Simeon, C.M. Rice, Z. Chen, A cell protection screen reveals potent inhibitors of multiple stages of the hepatitis C virus life cycle, Proc. Natl. Acad. Sci. U. S. A. 107 (2010) 3764–3769.

[26] P. de Medina, G. Favre, M. Poirot, Multiple targeting by the antitumor drug tamoxifen: a structure-activity study, Curr. Med. Chem. Anticancer Agents 4 (2004) 491–508.

[27] E.R. Prossnitz, J.B. Arterburn, L.A. Sklar, GPR30: a G protein-coupled receptor for estrogen, Mol. Cell. Endocrinol. 265–266 (2007) 138–142.

[28] P. Thomas, Y. Pang, E.J. Filardo, J. Dong, Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells, Endocrinology 146 (2005) 624–632.

[29] M.K. Dennis, R. Burai, C. Ramesh, W.K. Petrie, S.N. Alcon, T.K. Nayak, C.G. Bologa, A. Leitao, E. Brailoiu, E. Deliu, N.J. Dun, L.A. Sklar, H.J. Hathaway, J.B. Arterburn, T.I. Oprea, E.R. Prossnitz, In vivo effects of a GPR30 antagonist, Nat. Chem. Biol. 5 (2009) 421–427.