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Resistance to cyclosporin A derives from mutations in hepatitis C virus nonstructural proteins

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ABSTRACT

Cyclosporine A (CsA) is an immunosuppressive drug that targets cyclophilins, cellular cofactors that regulate the immune system. Replication of hepatitis C virus (HCV) is suppressed by CsA, but the molecular basis of this suppression is still not fully understood. To investigate this suppression, we cultured HCV replicon cells (Con1, HCV genotype 1b, FLR-N cell) in the presence of CsA and obtained nine CsA-resistant FLR-N cell lines. We determined full-length HCV sequences for all nine clones, and chose two (clones #6 and #7) of the nine clones that have high replication activity in the presence of CsA for further analysis. Both clones showed two consensus mutations, one in NS3 (T1280V) and the other in NS5A (D2292E). Characterization of various mutants indicated that the D2292E mutation conferred resistance to high concentrations of CsA (up to 2 μM). In addition, the missense mutation T1280V contributed to the recovery of colony formation activity. The effects of these mutations are also evident in two established HCV replicon cell lines—HCV-RMT ([1], genotype 1a) and JFH1 (genotype 2a). Moreover, three other missense mutations in NS5A—D2303H, S2362G, and E2414K—enhanced the resistance to CsA conferred by D2292E; these double or all quadruple mutants could resist approximately 8- to 25-fold higher concentrations of CsA than could wild-type Con1. These four mutations, either as single or combinations, also made Con1 strain resistant to two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811) or Debio-025. Interestingly, the changes in IC_{50} values that resulted from each of these mutations were the lowest in the Debio-025-treated cells, indicating its highest resistant activity against the adaptive mutation.

1. Introduction

The genome of the hepatitis C virus (HCV) is a single-stranded RNA with positive polarity and is classified in the *Flaviviridae* family. HCV frequently establishes chronic infections that lead to liver cirrhosis and hepatocellular carcinoma (HCC) [2]. An estimated 130–200 million people worldwide are now infected with HCV [3]. HCVs have been classified into six major genotypic groups (genotypes 1–6); genotype 1 is the most prevalent over most of the world. Treatments with alpha interferon (IFNα), together with the nucleoside analog ribavirin (RBV), greatly increased the percentage of HCV chronically infected patients able to reach a sustained anti-viral response (SVR). Covalent attachment of polyethylene glycol (PEGylated) IFN-α-plus-RBV therapy has a success rate of ~80% in patients with genotype 2 or 3 infections, but only ~50% in patients with genotype 1 infections [4,5]. The recently approved protease inhibitors boceprevir and telaprevir each improved the efficacy of IFN-α-plus-RBV therapy [6]. These direct-acting agents (boceprevir, simeprevir, sofosbuvir, faldaprevir and telaprevir, etc.) each have the advantage of being highly specific, but each may select for specific resistant mutations, limiting their long-time efficacy. Therefore, antiviral inhibitors targeting host factors crucial for viral replication should be developed to overcome these problems.

Abbreviations: HCV, hepatitis C virus; CsA, cyclosporine A; HCC, hepatocellular carcinoma; IFNα, alpha interferon; Cyp, cyclophilins; SVR, sustained anti-viral response.

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Reportedly, several HCV proteins interact with cyclophilins (Cyp) and modulate HCV replication [7–9]. To date, three Cyp inhibitors—Debio-025, NIM811, and SCY-635—have been deemed safe and effective for patients with HCV in phase I and II studies [10–12]. Development of Debio-025 has advanced the farthest through phase II studies, and Debio-025 has approved and showed a great deal of promise for decreasing HCV viremia in infected patients. However, emergence of drug-resistant HCV mutants could limit the therapeutic potential of CsA and Cyp inhibitors.

The HCV genome is a positive-sense, single-stranded RNA (about 9.6 kb) that encodes at least 10 viral proteins; these are categorized as structural core proteins (E1, E2) or nonstructural (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [13,14]. The nonstructural proteins are involved in HCV RNA replication [14]. NS5A protein comprises three domains linked by two low-complexity sequences (LCS) that are either serine or proline rich; domain I is a highly structured zinc binding domain whose three-dimensional structure shows two dimeric conformations [15,16]. Domains II and III have been shown to be unstructured in their native states, but nuclear magnetic resonance and circular dichroism have shown that elements of secondary structure run throughout each of these domains [17–19]. NS5A is anchored to membranes by an N-terminal amphipathic helix and is an essential component of the viral genome replication complex; it also interacts with other non-structural proteins [20] or cellular factors. NS5A domain II is a substrate for the peptidyl-prolyl cis/trans isomerase activity of Cys A and B [21], and NS5A domain III is reportedly a substrate of CypA [22].

In this study, we used CsA to select for and isolate drug-resistant HCV mutants; we then performed virus genome sequencing to investigate the molecular mechanisms of this drug resistance.

2. Materials and methods

2.1. Cells, electroporation and ethics statement

HuH-7 cells were cultured in DMEM-GlutaMax-I (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Replicon cells were maintained in the same medium supplemented with 300 μg/ml G418 (Invitrogen). Cells were passaged three times a week, and at each passage, each culture was split into four subcultures. Electroporation of replicon RNA and G418 selection were performed as previously described [23]. All experimental protocol was approved by the regional research institute.

2.2. Establishment of cyclosporin A resistant replicon clones

FLR3-1 cells derived from Con1 (AJ238799)-based, luciferase-harboring HCV sub-genomic replicon cell were treated with both 2 μM of cyclosporin A and 0.5 mg/ml of G418 for 24 days. Surviving cells were further treated with 3 μM CsA for 2 days, 4 μM for 4 another days, and finally 6 μM for the last 10 days. Using limiting dilution cloning, we established nine clonal cell lines. Using real-time RT-PCR (ABI 7700 system, Applied Biosystems, Foster City, CA, USA) as described previously [24], we systematically measured HCV RNA copy number in each of these nine clonal lines.

2.3. Determination of consensus sequence of resistant clones

LongRange Reverse transcriptase (QIAGEN, Valencia, CA, USA) and an oligonucleotide primer (antisense sequence 9549–9569 of HCV-Con1) were used to reverse transcribe purified RNA (1 μg). The resulting cDNA, Phusion DNA polymerase (Finnzymes, Vantaa, Finland), and primers recognizing each non-coding region were used for PCR amplification of the entire non structural protein coding region of the sub-genomic replicon. The TA cloning kit (Invitrogen) was used to introduce each fragment into a separate plasmid; we picked up eight clones from each resistant cell line and their nucleotide sequences were determined.

2.4. Construction and RNA transcription

The pFK I389neo/NS3-3′/5.1 and pFK I389luc/NS3-3′/5.1 plasmids (ReBlikon, Baden-Württemberg, Germany) were used to generate HCV constructs with regions of the sub-genomic replicon with mutations (Fig. 2A). The QuikChangeII kit (Stratagene, La Jolla, CA, USA) was used to introduce specific mutations into the HCV sequences. To generate RNA, plasmids were digested with *Xba*I and used as a template for RNA transcription; RiboMax (Promega, Madison, WI, USA) was used for each transcription reaction.

2.5. Drug treatment

For the drug resistance assays, established CsA-resistant replicon clones were seeded onto 24-well tissue culture plates (10,000 cells/well) and cultivated overnight. Then cells were treated with various concentrations of CsA (0–8 μM) for 4 days. Surviving cells were stained with crystal violet.

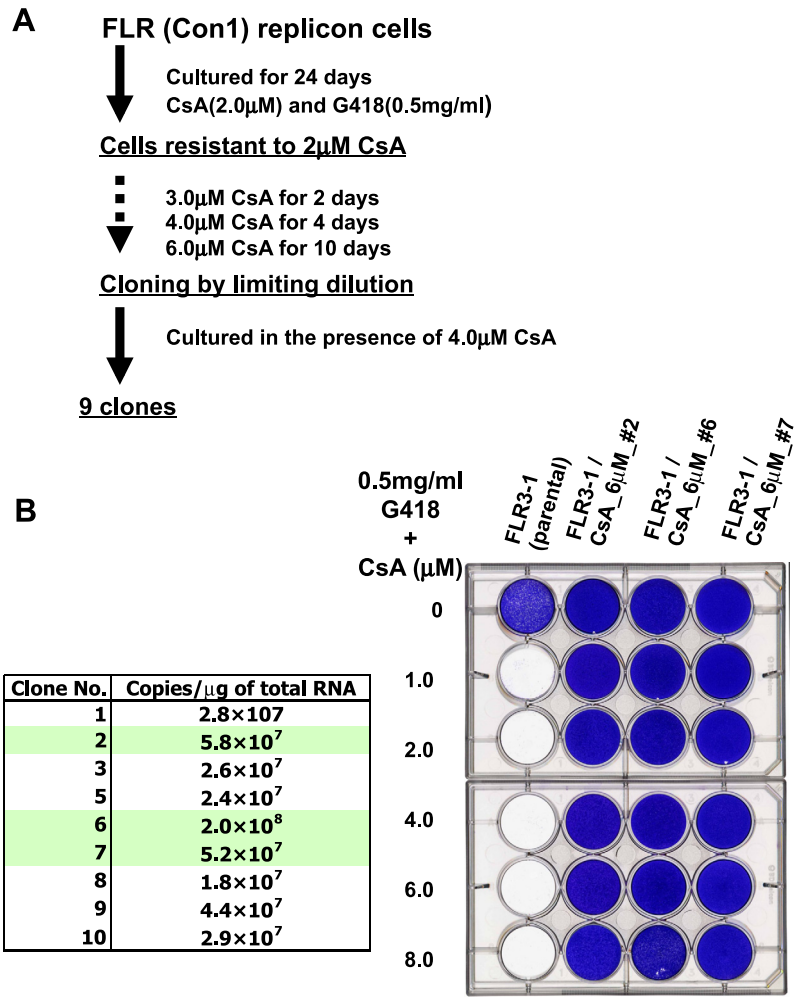
For HCV replication inhibition assays, replicon cells were seeded in 96-well tissue culture plates (5000 cells/well) and cultivated overnight. Serial dilutions of CsA (Fluka Chemie, Buchs, Switzerland) or NIM811 (Novartis) and Debio025 (Debiopharma) were then added to sets of wells. After incubation for 72 h, ABI prizm 6100 (Applied Biosystems) was used to extract total RNA from cells, and HCV-RNA was measured as described above. Each assay was carried out in triplicate.

For another HCV replication inhibition assay, mutant replicon RNA derived from pFK I389luc/NS3-3′/5.1 plasmid were introduced into HuH7 cells via electroporation, and the transformed/transfected cells were seeded to 96-well tissue culture plates. Drugs were added 24 h after electroporation. Luciferase activities were evaluated 4 h or 72 h after electroporation, which corresponded to 20 h before drug treatment or 48 h after drug treatment, respectively; the Blight-Glo kit (Invitrogen) and Envision (Perkin Elmer, Waltham, MA, USA) were used to take all measurements, and values at 72 h were normalized relative to the values from 4 h.

3. Results

3.1. Establishment of CsA-resistant clones

To establish CsA-resistant clones, we treated HCV FLR-N replicon cells with CsA (Fig. 1A) and obtained nine resistant clonal cell lines. We measured the amount of HCV RNA in each resistant clonal line and chose for further study the three lines that consistently had the largest amount of HCV RNA (Fig. 1B). We then determined the entire HCV sequence from 16 subclones; we isolated two groups of eight subclones (one group each from clones #6 and #7), because we could not establish clone #2; each subclone was isolated by treating a CsA-resistant clone (#6 or #7) with 6 μM CsA (Table 1). Although there were several mutations in the NS3–NS5B protein-coding regions, common mutations were isoleucine (I) to valine (V) at amino acid 1280 (T1280V) and aspartic acid (D) to glutamic acid (E) at amino acid 2292 (D2292E). At 1280, original Con1 has threonine (T) and was mutated into (I) in Con1 replicon cells.



Clone No.	Copies/μg of total RNA
1	2.8×107
2	5.8×10^{7}
3	2.6×10^{7}
5	2.4×10^{7}
6	2.0×10^{8}
7	5.2×10^{7}
8	1.8×10^{7}
9	4.4×10^{7}
10	2.9×10^{7}

Fig. 1. Basic characteristics of the nine cyclosporin A-resistant clones. (A) Flow chart outlining the selection of cyclosporin A-resistant HCV replicon clones. (B) Real-time PCR was used to determine the copy number of each Cys A-resistant clone. The three clones with the highest HCV genome copy number are highlighted in green (Left). Colony formation assay of mutant #2, 6 and 7 (Right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. Identification of mutations responsible for CsA resistance

To define the mutations responsible for CsA resistance, we constructed various chimeric clones that each contained specific mutation that arose from CsA selection (Fig. 2A). We could thereby evaluate each mutation with regard to its effect on CsA resistance. We found that mutations in two proteins—NS5A and NS4A—significantly enhanced the resistance against CsA treatment (Fig. 2B). We also cultured replicon cells with these mutants in the presence of CsA (up to 2 μM); we found that cells with a D2292E mutation could survive, but cells with wild-type NS5A or T1280V mutation could not (Fig. 3A).

The effect of T1280V mutation on colony formation was further evaluated (Fig. 3B). Introduction of the T1280V mutation in *cis* to the D2292E mutation rescued the colony-formation defect of the D2292E mutant replicon cells; specifically, the T1280V–D2292E double-mutant replicon cells had the same colony-forming ability as the parental replicon cells.

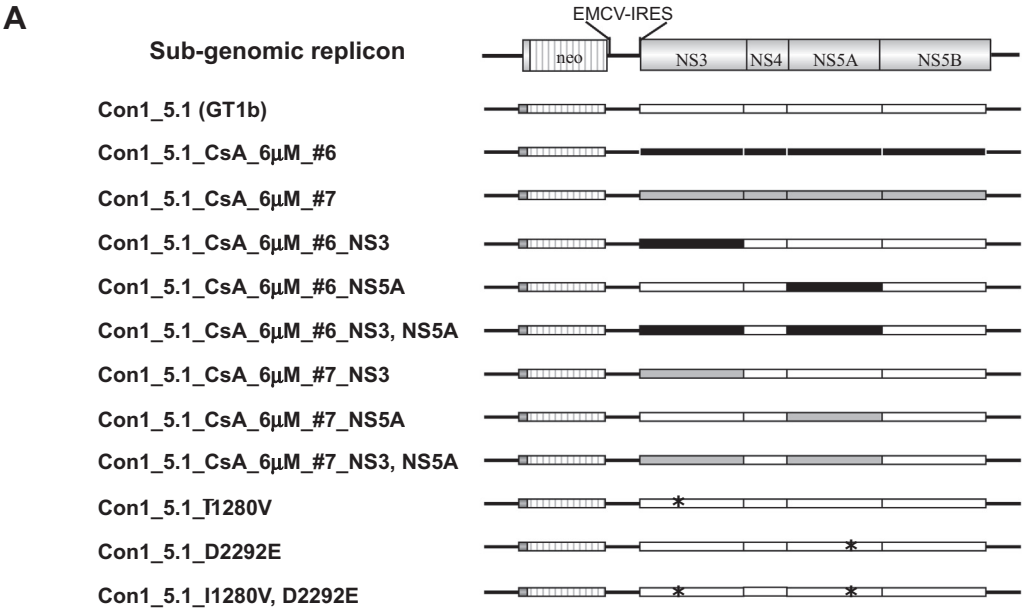
3.3. Evaluation of mutations for CsA resistance in other HCV genotypes

We evaluated whether the mutations that conferred CsA resistance to the HCV Con1 strain (genotype 1b) also conferred CsA resistance to the RMT (genotype 1a; AB520610) and JFH1 (genotype 2a; AB047639) strains (Fig. 4A, B and Table 2) [1]. D2292E conferred CsA resistance to the HCV strains RMT and JFH1, but T1280V did not (Table 2), as observed with HCV Con1 strain (Fig. 2E). The amino acid sequences surrounding mutations other than D2292E showed some differences among three genotypes (1a, 1b, and 2a) (Fig. 4B). D2292E mutants of these three genotypes showed resistance to CsA (Fig. 2E, Table 2) but the fold increase of resistance in genotype 1a and 2a was lower than that of genotype 1b (Tables 2 and 3). Therefore, there might be some residue(s) other than D2292E to influence the resistance to CsA.

3.4. Efficacy of mutations in NS5A for conferring CsA resistance

Although D2292E clearly conferred CsA resistance to HCV, other mutations in NS5A may also have had an effect because constructs with all four of the original NS5A mutations found in clone #6 mutations were more resistant to CsA than were constructs with only the D2292E mutation (Fig. 2B and E). We constructed HCV-luciferase replicons, each with one or more of four mutations (D2292E, D2303H, S2362G, and E2414K). HuH-7 cells were transiently transfected with RNA of each construct; we then treated the transfected cells with CsA (Table 3). Of the four single mutants, all but S2362G conferred some CsA resistance to HCV-luciferase replicons; notably, combinations of mutations had additive effects

A



B

Cyclosporin A	Replacement or Mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.18	1.0
	Cs6#6	-	-	0.21	1.2
	-	-	Cs6#6	2.10	11.7
	Cs6#6	-	Cs6#6	2.20	12.2
	Cs6#6	V1681A	Cs6#6	5.40	30.0

D

Debio025	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.05	1.0
	Cs6#6	-	-	0.04	0.8
	-	-	Cs6#6	0.43	7.8
	Cs6#6	-	Cs6#6	0.40	8.0
	Cs6#6	V1681A	Cs6#6	0.75	13.7

C

NIM811	replacement or mutations			IC_{50} (μM)	Fold change
	NS3	NS4A	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.09	1.0
	Cs6#6	-	-	0.10	1.1
	-	-	Cs6#6	0.49	5.4
	Cs6#6	-	Cs6#6	0.50	5.6
	Cs6#6	V1681A	Cs6#6	1.80	20.0

E

Cyclosporin A	Replacement or mutation			IC_{50} (μM)	Fold change
	NS3	NS4B	NS5A		
Con1_5.1 (GT1b)	-	-	-	0.54	1.0
	Cs6#7	-	-	0.59	1.1
	-	-	Cs6#7	2.36	4.4
	Cs6#7	-	Cs6#7	2.51	4.6
	Cs6#7	I1797V	Cs6#7	2.44	4.5
	T1280V	-	-	0.57	1.1
	-	-	D2292E	1.71	3.1
	T1280V	-	D2292E	1.76	3.3

Fig. 2. (A) Schematic representations of 12 Con1 replicon-derived constructs. (B–D) Evaluation of Cs6#6 constructs with regard to resistance to CsA or to each of two CsA derivatives (NIM811 and Debio025). Real-time PCR was used to measure HCV sub-genome copy number in cells, and IC_{50}s were then determined from the copy number values. For each construct, the fold change represents the ratio of IC_{50} values from the construct and the parental Con1 replicon (IC50Construct:IC50Parental). (E) Resistance to CsA of three Cs6#7 derivative constructs that represent the T1280V and D2292E mutations as each single mutation or as a double mutation.

and conferred greater CsA resistance than any single mutation. The HCV replicon with all four mutations showed the strongest CsA resistance.

3.5. Evaluation of CsA-resistant mutants for resistance to cyclophilin inhibitors

We further evaluated each of the NS5A mutants for their ability to confer resistance to each of two other cyclophilin inhibitors, N-methyl-4-isoleucine-cyclosporin (NIM811, Table 4) and Debio-025 (Table 5). Of the four single mutants, D2292E conferred the highest resistance, and the combination of all four mutations conferred the overall highest resistance to NIM811 and to Debio-025. When we compared CsA, NIM811, and Debio-025, the mutation-mediated increases in IC_{50} values were lowest with the Debio-025 treatment (Tables 3–5).

4. Discussion

Here, we investigated two of nine HCV sub-genomic replicon cell clones (CsA-resistant HCV mutants) isolated following long-term dual treatment with CsA and G418. Comparing the HCV sequences of these two clones (#6 and #7), only two of many mutant sites were shared between the mutant HCV sequences. Specifically, both clones #6 and #7 had a D2292E missense mutation in NS5A and a T1280V missense mutation in NS3. D2292E is

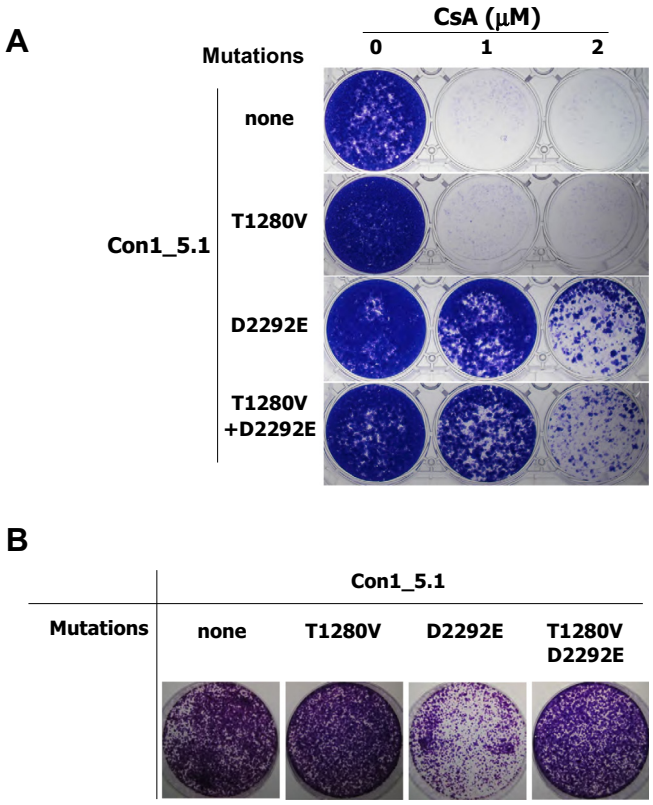


Fig. 3. (A) Resistance to CsA of T1280V and D2292E mutants. While under G418 selection, established replicon cells were treated with CsA at the indicated doses. (B) Standard methods described in Section 2 were used to determine the colony-forming abilities of T1280V and D2292E mutants.

known to confer CsA resistance to some HCV genotypes [25–28], and as a single mutation, it conferred CsA resistance to three separate HCV strains in our hands. In contrast, T1280V in NS3 was not previously identified as a CsA-resistance mutant, and in our hands, it had no impact on CsA resistance as a single mutation (Figs. 2E and 3A).

D2292E was the most significant resistance mutation in this study (Fig. 4C). This mutation is also significant in the regulation of HCV genome replication [29], and close to the CypA binding region [30] (Supplementary Fig. 1). With several genotypes (1a, 1b, 2a, 3, 4, and 6), D2292E is frequently observed after Debio-025 selection [28,31]. Other different mutations in NS5A and NS5B were identified in other studies of CsA resistance [7]; therefore, various mutations could influence HCV resistance to CsA.

In addition to the D2292E mutation, the T1280V mutation in NS3 was present in both clones #6 and #7. Despite its presence in both clones, it did not confer CsA resistance as a single mutant, nor did it enhance the effects of the NS5A CsA-resistant mutants (Fig. 2E). Instead, it partially rescued the colony-forming defect caused by D2292E (Fig. 3B). We used three assays—colony formation assay without CsA treatment (Fig. 3B), cell survival assay of established replicon cells with CsA and G418 dual-treatment (Fig. 3A), and HCV replication inhibition assay without G418 treatment (Fig. 2E and Table 2)—to evaluate the HCV replication competence of each of these two mutations (D2292E, T1280V). It is difficult to fully explain all of the results, and comparison of the two CsA-resistant clones (clone #6 and #7) leaves some questions unanswered. These clones were similar to each other when considering survival during CsA and G418 dual-treatment (Fig. 1B), but they show differences in their resistance in HCV sub-genome replication assay (Fig. 2B and E). Apparently, each mutation in clone #7, except for D2292E, had no effect on the results of the HCV sub-genome replication inhibition assay with CsA. These findings might suggest that these mutations were important to G418 resistance, but not to the resistance of HCV to CsA treatment. In contrast, each of three other mutations in NS5A (D2303H, S2362G, and E2414K) that were found in clone #6 were required for the maximum level of drug resistance conferred by a mutant NS5A in this study. To our knowledge, D2303H is a novel CsA-resistant mutation, and as a single mutation, it conferred CsA resistance comparable to D2292E. D2303H, like D2292E, was located in carboxy-terminal of domain II of NS5A, which is reportedly a CypA binding site [9]. S2362G and E2414K were mutations in domain III of NS5A, and these mutations may have influenced the peptidyl-prolyl isomerase enzymatic catalytic activity of CypA [22]. The V1681A mutation in NS4A identified in clone #6 greatly enhanced the CsA resistance of a HCV construct that had NS3 and NS5A replaced with Cs6#6 sequences (Fig. 2B–D). Though

Table 1
The list of each mutated amino acid sequences in 16 clones throughout whole non-structural region.

		NS3						4A	4B	NS5A										5B
A.A. No.		1062	1275	1280	1560	1609	1612	1681	1797	2109	2179	2197	2231	2269	2292	2303	2320	2362	2387	2414	2992
p5.1		V	D	T	S	K	I	V	I	D	S	P	L	S	D	D	K	S	S	E	M
Cs6#6	1	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	D	D	K	**G**	S	**K**	M
	2	V	D	**V**	S	**E**	I	V	I	**N**	S	P	L	S	D	D	K	S	S	E	M
	3	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	4	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	5	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	6	V	D	**V**	**G**	K	**T**	**A**	I	**E**	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	7	V	D	**V**	**G**	K	**T**	**A**	I	**E**	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
	8	V	D	**V**	**G**	K	**T**	**A**	I	D	S	P	L	S	**E**	**H**	K	**G**	S	**K**	M
Cs6#7	1	**I**	**G**	**V**	S	**E**	I	V	I	**N**	S	P	**P**	**P**	**E**	D	K	S	P	**G**	**T**
	2	V	D	**V**	S	**E**	I	V	I	**N**	S	P	**P**	**P**	**E**	D	K	S	P	**G**	**T**
	3	**I**	**G**	**V**	S	K	I	V	**V**	**N**	**P**	**L**	L	S	**E**	D	**T**	S	S	E	M
	4	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	S	**E**	D	**M**	S	S	E	M
	5	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	S	**E**	D	**T**	S	S	E	M
	6	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	S	**E**	D	**T**	S	S	E	**T**
	7	**I**	**G**	**V**	S	K	I	V	**V**	D	**P**	**L**	L	**P**	**E**	D	K	S	P	**G**	**T**
	8	V	D	**V**	S	**E**	I	V	I	**N**	S	P	**P**	**P**	**E**	D	K	S	P	**G**	**T**

The two gray-highlighted lines were selected as the representative sequences of CsA_6μM_#6 and #7 and used to generate the derivative constructs.

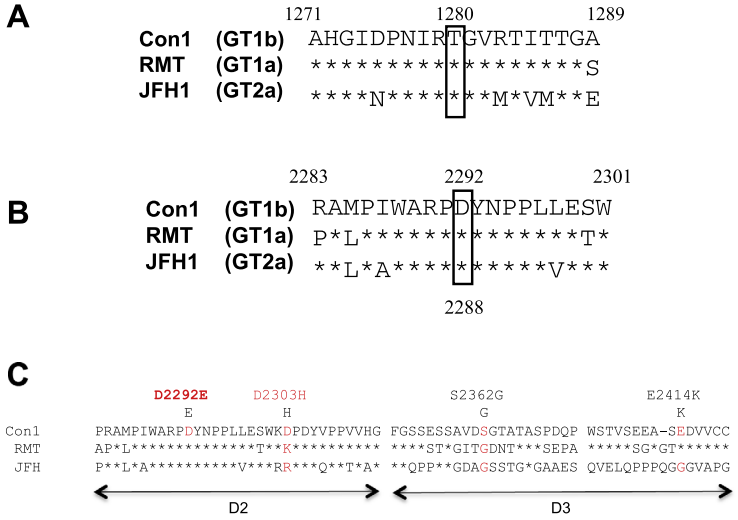


Fig. 4. Amino acid sequences of HCV-RMT-tri (GT1a) and HCV-JFH1 (GT2a) around (A) T1280V and (B) D2292E. (C) Location of the CsA resistant mutations in NS5A. Amino acid sequences around the positions of four CsA resistant mutations.

Table 2
Evaluation of resistance to CsA of mutants that have single mutations or combinations of multiple mutations.

	Mutations		IC_{50} (μm)	Fold change
	NS3	NS5A		
RMT-tri (RMT, GT1a)	–	–	0.79	1.0
	–	D2292E	2.1	2.7
	T1280I	–	0.96	1.2
	T1280I	D2292E	2.46	3.1
	T1280V	–	0.91	1.2
	T1280V	D2292E	2.54	3.2
JFH1 (JFH1, GT2a)	–	–	0.49	1.0
	–	D2292E	1.3	2.7
	T1280I	–	0.51	1.0
	T1280I	D2292E	1.38	2.8
	T1280V	–	0.69	1.4
	T1280V	D2292E	1.2	2.4

Threonine at site 1280 (RMT-tri or JFH1) were mutated to isoleucine (adaptive mutation of Con1 replicon) or valine (major mutation of CsA resistant clones). Aspartic acid at 2292 was mutated to glutamic acid.

Table 3
Evaluation of amino acid mutations in NS5A that conferred CysA resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.11	1.0
	○				0.88	7.9
		○			0.52	4.7
			○		0.12	1.0
				○	0.30	2.7
		○	○	○	1.0	9.4
	○	○			1.8	16.6
	○		○		0.95	8.5
	○			○	1.5	13.1
	○	○	○	○	2.80	25.7

we have not assessed V1681A as single mutant, analyzing its mechanism of CsA resistance and its cooperation with other mutations in NS3 and NS5A must be worthwhile because V1681A greatly enhanced the CsA resistance of some constructs.

In all, we evaluated three cyclophilin inhibitors—CsA, NIM811, and Debio-025. Among them, Debio-025 showed the strongest inhibition (IC_{50} values to any mutants) and was tolerated by CsA-resistant mutations (IC_{50} index change values, Fig. 2 and

Table 4
Evaluation of amino acid mutations in NS5A that conferred NIM811 resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.054	1.0
	○				0.324	6.0
		○			0.184	3.4
			○		0.056	1.0
				○	0.125	2.3
		○	○	○	0.455	8.4
	○	○			0.635	11.8
	○		○		0.403	7.5
	○			○	0.599	11.1
	○	○	○	○	0.923	17.1

Table 5
Evaluation of amino acid mutations in NS5A that conferred Debio-025 resistance.

	Mutations in NS5A				IC_{50} (μm)	Fold change
	D2292E	D2303H	S2362G	E2414K		
Con1_5.1 (GT1b)					0.024	1.0
	○				0.095	4.0
		○			0.074	3.1
			○		0.028	1.2
				○	0.024	1.8
		○	○	○	0.139	5.8
	○	○			0.198	8.3
	○		○		0.139	5.8
	○			○	0.185	7.8
	○	○	○	○	0.263	11.0

Table 3–5). It was interesting that the resistant mutants differed so greatly in their tolerance of these three inhibitors because all three inhibitors have the same mode of action. Garcia-Rivera et al. concluded that CsA resistance of HCV mutants were solely derived from dependence of the NS5A proteins on cyclophilins [28]. Our results might indicate that other factors are important to CsA resistance, in addition to residual cyclophilin activity.

Drugs that are intended to treat chronic HCV infection and that target important nonstructural HCV proteins—the serine protease NS3/4A, the large phosphoprotein NS5A, or the RNA-dependent RNA polymerase NS5B—have reached the clinical trial stage of drug development [32–34]. Two oral HCV protease inhibitors were approved by the FDA, and some of the drugs could achieve a sus-

tained virologic response (SVR) [35]. However, to develop treatments that eradicate individual chronic HCV infections, additional studies on the emergence of drug-resistant HCV mutants and on the molecular interactions at HCV replication complexes are necessary.

Our new findings provided insights into the way by which HCV acquires resistance to cyclophilin inhibitors, and these insights will facilitate the development of this type of anti-HCV drug for clinical use.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.04.053.

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