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LIVER

A Translational Study of Resistance Emergence Using Sequential Direct-Acting Antiviral Agents for Hepatitis C Using Ultra-Deep Sequencing

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OBJECTIVES: **Direct-acting antiviral agents (DAAs) against hepatitis C virus (HCV) have recently been developed and are ultimately hoped to replace interferon-based therapy. However, DAA monotherapy results in rapid emergence of resistant strains and DAAs must be used in combinations that present a high genetic barrier to resistance, although viral kinetics of multidrug-resistant strains remain poorly characterized. The aim of this study is to track the emergence and fitness of resistance using combinations of telaprevir and NS5A or NS5B inhibitors with genotype 1b clones.**

METHODS: **HCV-infected chimeric mice were treated with DAAs, and resistance was monitored using direct and ultra-deep sequencing.**

RESULTS: **Combination therapy with telaprevir and BMS-788329 (NS5A inhibitor) reduced serum HCV RNA to undetectable levels. The presence of an NS3-V36A telaprevir resistance mutation resulted in poor response to telaprevir monotherapy but showed significant HCV reduction when telaprevir was combined with BMS-788329. However, a BMS-788329-resistant strain emerged at low frequency. Infection with a BMS-788329-resistant NS5A-L31V mutation rapidly resulted in gain of an additional NS5A-Y93A mutation that conferred telaprevir resistance during combination therapy. Infection with dual NS5AL31V/NS5AY93H mutations resulted in poor response to combination therapy and development of telaprevir resistance. Although HCV RNA became undetectable soon after the beginning of combination therapy with BMS-788329 and BMS-821095 (NS5B inhibitor), rebound with emergence of resistance against all three drugs occurred. Triple resistance also occurred following infection with the NS3V36A/NS5AL31V/NS5AY93H triple mutation.**

CONCLUSIONS: **Resistant strains easily develop from cloned virus strains. Sequential use of DAAs should be avoided to prevent emergence of multidrug-resistant strains.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

According to the 2012 World Health Organization report, approximately 150 million individuals are chronically infected with hepatitis C virus (HCV) worldwide (1). Chronic HCV infection leads to chronic hepatitis, liver cirrhosis, liver failure, and hepatocellular carcinoma (1). Recently, two direct-acting antiviral agents (DAAs), telaprevir and boceprevir, have been approved for use in daily clinical practice to treat patients chronically infected with HCV genotype 1 (2–9). Triple therapy with peg-interferon, ribavirin, and either telaprevir or boceprevir has been reported to be the most effective approved treatment so far, with an eradication rate of 50 to 70%, compared with no >50% for combination therapy with peg-interferon and ribavirin alone (2–9). However, triple therapy is approved only for genotype 1, and many treated patients experience severe side effects that often result in early termination of the therapy (2–9). In an effort to establish safer

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and more effective therapies, a number of new DAAs are in development, and several have reached the clinical trial stage (10–12).

One of the two available drugs, telaprevir, has been approved in the United States, Canada, the Europe Union, and some Far East countries. Although telaprevir, a potent NS3/4A protease inhibitor, shows excellent antiviral activity, single use of the drug results in rapid emergence of resistant mutants (13–16). Twelve weeks of monotherapy with telaprevir resulted in a marked reduction of HCV RNA (16), and HCV RNA became undetectable in the serum in 3 of 10 treated patients (16). However, emergence of resistant mutants caused viral breakthrough and reappearance of the virus. To date, only one patient who showed a sustained virological response has been reported (16–18).

To avoid the emergence of resistance, combinations of DAAs targeting different viral protein with or without ribavirin have been investigated (19–22). We previously showed in animal experiments that HCV can be eradicated by only 4 weeks of combination therapy with telaprevir and NS5B inhibitor MK0608 (19). More recently, the combination of the NS3 protease inhibitor asunaprevir with the NS5A inhibitor daclatasvir was shown to eradicate the virus successfully in patients with genotype 1b (21,22). This interferon-free regimen has been reported to be more tolerable with few severe adverse events. Only some patients developed an elevation of transaminases and hyperbilirubinemia, probably due to the side effects of asunaprevir, which selectively accumulates in the liver with liver vs. plasma ratios ranging from 40-fold to 359-fold in several animal species (23). It has been reported that daclatasvir is effective even with very small doses and that the drug is excreted from the kidneys, suggesting that the liver damage seen in the combination therapy is not due to daclatasvir (22). As a significant portion of patients with HCV infection have already developed advanced liver diseases, the risk of coercing further liver damage should be minimized. Although it has been reported that telaprevir affects both liver and kidney transporters (24), no significant liver damage by telaprevir has been reported so far. This suggests that telaprevir is a good candidate for a future oral DAA combination therapy with daclatasvir.

We investigated in this study the effect of BMS-788329, a close analog of daclatasvir and telaprevir combination therapy against HCV genotypes 1b using a human hepatocyte chimeric mouse infection model. We also assessed how existing variants with resistant features affect response to DAA combination therapy. Furthermore, we investigated the possibility of multiple drug-resistant mutants using the combination of BMS-788329 and polymerase inhibitor BMS-821095. Our results showed that a mutant strain resistant against all three drugs emerges from clonal infection after sequential therapy with these drugs. Therefore, our results advocate for simultaneous DAA combination therapy, and we note the importance of resistance analysis and drug selection before therapy in order to successfully eradicate the virus.

METHODS

Animals

Generation of the uPA+/+/SCID+/+ mice and transplantation of human hepatocytes were performed as described previously (25). All mice were transplanted with frozen human hepatocytes obtained from the same donor. Animal protocols were approved by and performed in accordance with the guidelines of the local committee for animal experiments. Mice received humane care. Infection, extraction of serum samples, and killing were performed under ether anesthesia. Mice were inoculated intravenously with HCV-positive human serum samples and used for evaluation of drugs as reported previously (26,27). Mice were also prepared by injection of genotype 1b clone HCV-KT9 HCV RNA and its synthesized derivatives into the livers of chimeric mice (28).

Reagents

HCV-infected mice were perorally administered telaprevir based on 200 mg/kg body weight of (VX950; MP424; Mitsubishi Tanabe Pharma, Osaka, Japan), 10 mg/kg body weight of BMS-788329 (NS5A inhibitor), and 100 mg/kg body weight of BMS-821095 (NS5B inhibitor) (Bristol-Myers Squibb, New York, NY). These reagent doses were found to yield serum concentrations equivalent to those in treated human patients.

Human serum samples

Human serum samples containing a high titer of genotype 1b HCV (2.2×10^6–10^7 copies/ml) were obtained from patients with chronic hepatitis C after obtaining written informed consent. Aliquots of serum were stored in liquid nitrogen until use. The study protocol involving human subjects conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

Construction of mutant HCV strains and HCV RNA synthesis

We have previously reported infectious genotype 1b HCV clone HCV-KT9, which is able to replicate in human hepatocyte chimeric mice (27) (GenBank accession no. AB435162). HCV-KT9 mutants (V36A in NS3, L31V and Y93H in NS5A) were generated by site-directed mutagenesis using following primers: V36A in NS3, 5′-AGGTTCAAATAGCCTCCACCGCAACA-3′ (sense) and 5′-TGTTGCGGTGGAGGCTATTTGAACCT-3′ (antisense); L31V in NS5A, 5′-TCCAAACTCCTGCCGCGGGTACCGGGA GTCCCTTT-3′ (sense) and 5′-AAAGGGACTCCCGGTACCCG CGGCAGGAGTTTGGA-3′ (antisense); and Y93H in NS5A, 5′-AACATTCCCCATCAACGCACACACCACGGGCCCCTG CACA-3′ (sense) and 5′-TGTGCAGGGGCCCGTGGTGTGTGC GTTGATGGGGAATGTT-3′ (antisense). HCV RNA synthesis was performed as described previously (26,29). The RNA was analyzed using denaturing agarose gel electrophoresis and stored at −80 °C until use.

RNA extraction and HCV RNA quantification

RNA was extracted from mouse serum samples using Sepa Gene RV-R (Sankojunyaku, Tokyo, Japan), dissolved in RNase-free water, and reverse transcribed using a random primer (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) in a 20 µl reaction mixture according to the instructions provided by the manufacturer.

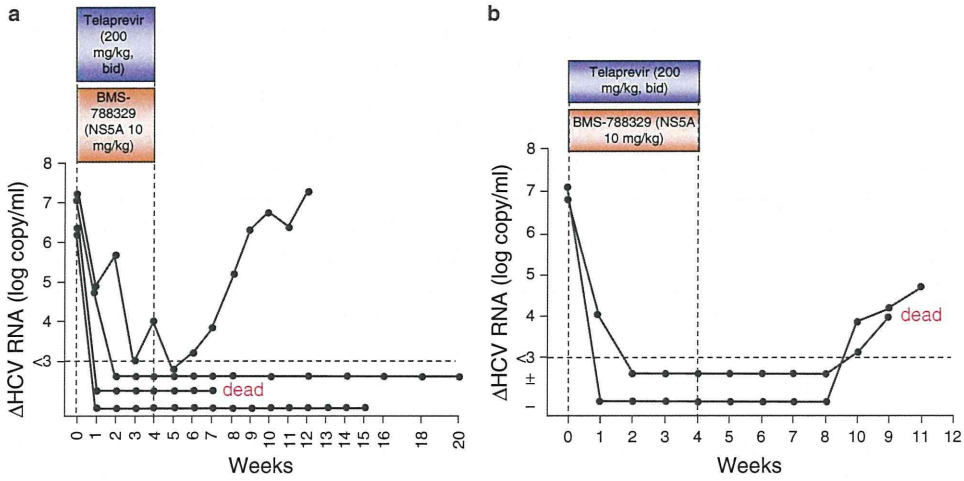

Figure 1. Effect of telaprevir plus NS5A inhibitor combination therapy for human hepatocyte chimeric mice infected with HCV genotype 1b. We established mice infected with serum from two different patients with genotype 1b and administrated 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). (**a**) Viral breakthrough occurred in one mouse infected with serum from the first patient. (**b**) Viral relapse occurred 5 weeks following the end of therapy in both mice infected with serum from the second patient. HCV, hepatitis C virus.

One microliter of complementary DNA were subjected to quantification of HCV RNA using 7300 Real-Time PCR System (Life Technologies, Carlsbad, CA) (27).

Ultra-deep sequencing

We amplified 200–300-bp HCV complementary DNA fragments using KOD DNA Polymerase by nested PCR using the following primers: NS3 first set, 5′-CTGCATCATCACTAGCCTTACG-3′ (sense) and 5′-GAGCACCTTGTACCCTTGGGC-3′ (antisense); NS5A first set, 5′-ACTACGTGCCTGAGAGCGACG-3′ (sense) and 5′-CCAACCAGGTACTGATTGAGC-3′ (antisense); NS3 aa36, 5′-AGAACCAGGTCGAGGGAGAGG-3′ (sense) and 5′-AAGTAGAGGTCCGAGCTGCCG-3′ (antisense); NS3 aa155-156, 5′-GGGCACGTTGTGGGCATCTTC-3′ (sense) and 5′-GAGCACCTTGTACCCTTGGGC-3′ (antisense); NS5A aa31, 5′-TGGCTCCAGTCCAAACTCCTG-3′ (sense) and 5′-GGGAATGTTCCATGCCACGTG-3′ (antisense); NS5A aa93, 5′-TGGAACATTCCCCATCAACGC-3′ (sense) and 5′-CCAACCAGGTACTGATTGAGC-3′ (antisense) and then we performed end repair of fragmented DNA, adenine tailing of end repair, adaptor ligation, and PCR enrichment of adaptor-ligated DNA using TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA) according to the instructions provided by the manufacturer. Paired-end sequencing with multiplexed tags was carried out using Illumina Genome Analyzer IIx.

Direct sequencing

To compare the results of direct and ultra-deep sequencing, we performed direct sequencing using the same DNA fragments as ultra-deep sequencing. The primers for NS5B were as follows: NS5B first set, 5′-CGTCTGCTGCTCAATGTCCTAC-3′ (sense) and 5′-GTCATGCGGCTCACGGACCT-3′ (antisense); NS5B second set, 5′-GACTCAACGGTCACTGAGAG-3′ (sense) and 5′-CCTATTGGCCTGGAGTGTTT-3′ (antisense). Direct sequencing was carried out using a 3130 Genetic Analyzer (Life Technologies).

RESULTS

Effect of telaprevir plus NS5A inhibitor combination therapy for human hepatocyte chimeric mice infected with HCV genotype 1b

We inoculated six human hepatocyte chimeric mice with serum samples obtained from two patients with genotype 1b. After HCV RNA levels reached plateau, mice were administrated 200 mg/kg of telaprevir and 10 mg/kg of BMS-788329 (NS5A inhibitor) for 4 weeks (**Figure 1**). HCV RNA levels of three out of the four mice with serum from patient 1 decreased below the limit of detection (1.0×10^3 copies/ml). HCV RNA levels of the fourth mouse flared up before the end of therapy (viral breakthrough), and HCV RNA levels rapidly returned to pre-treatment levels following the end of therapy (**Figure 1a**). In the two mice inoculated with serum from patient 2, HCV levels remained negative for 4 weeks after drug withdrawal in both mice and then gradually increased to 1.0×10^5 copies/ml (**Figure 1b**). These results indicate that telaprevir plus NS5A inhibitor combination therapy at the above dose is effective against HCV genotype 1b (**Figure 1a,b**).

Combination treatment with telaprevir and BMS-788329 in human hepatocyte chimeric mice infected with an HCV clone containing NS3 V36A telaprevir resistance mutation

We established clonal infection with a telaprevir-resistant NS3 V36A mutant KT-9 strain in two human hepatocyte chimeric mice. Mice were treated with telaprevir alone for the first 2 weeks to confirm resistance and then treated with telaprevir plus

BMS-788329 combination therapy thereafter. HCV RNA levels decreased only slightly in two mice when treated with telaprevir alone, indicating that the introduced NS3 V36A mutation conferred resistance against telaprevir. HCV RNA levels declined to undetectable levels in one of the mice (**Figure 2a**) and hovered near the limit of detection in the other mouse (**Figure 2b**).

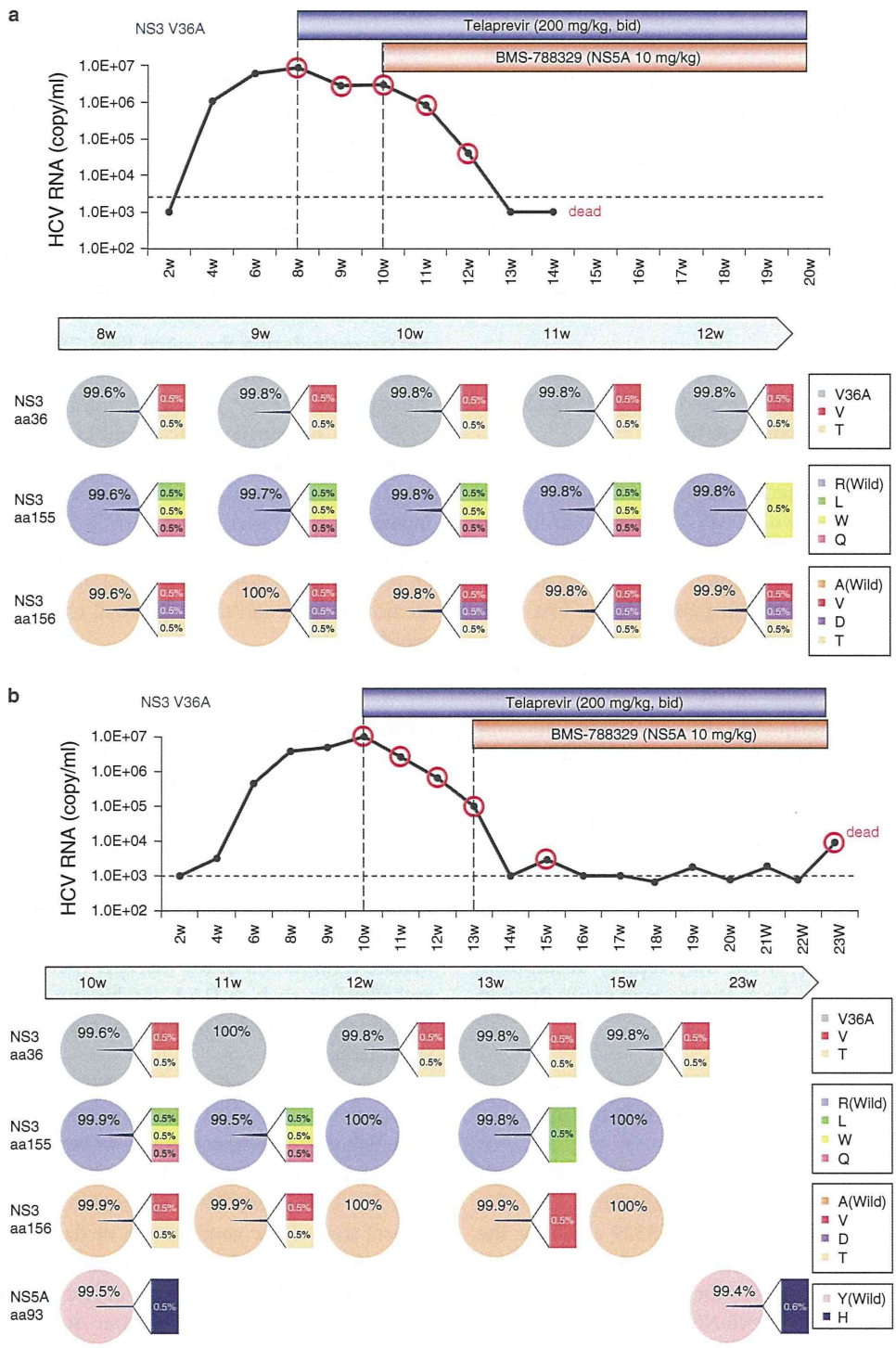

Figure 2. Combination treatment with telaprevir and BMS-788329 in human hepatocyte chimeric mice infected with an HCV clone containing NS3 V36A telaprevir resistance mutation. We infected mice with an infectious clone harboring a telaprevir-resistant NS3 V36A mutation. (**a**, **b**) Mice received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). HCV, hepatitis C virus; w, weeks.

Ultra-deep sequencing data showed that the introduced V36A mutation in the NS3 region of KT-9 was conserved in >99.5% of the viral sequences examined. In addition, amino acids 155 and 156 in NS3, which are also associated with telaprevir resistance, also remained unchanged (**Figure 2a,b** and **Supplementary Tables 1** and **2** online). Although mutant sequences were detected at very low frequency (<0.5%), these sequences may be due to sequencing errors or artifacts introduced during the amplification step because similar low-frequency variants were detected when sequencing a plasmid to establish the error threshold for detection of rare variants (data not shown). However, we detected a small amount of a new resistance mutation, Y93H in the NS5A region, in mice treated with telaprevir and BMS-788329 combination therapy for 10 weeks (**Figure 2b** and **Supplementary Table 2** online). These data indicate that sequential administration of telaprevir and NS5A inhibitor may result in emergence of a doubly resistant strain.

Effect of telaprevir and BMS-788329 combination therapy in human hepatocyte chimeric mice infected with an HCV clone containing NS5A L31V resistance mutation

We established clonal infection with an HCV KT-9 NS5A L31V mutant clone, which we expected to be resistant against NS5A inhibitor. Mice were treated with BMS-788329 alone for the first 2 weeks to confirm resistance and then treated with telaprevir plus BMS-788329 combination therapy thereafter. In both mice, HCV RNA levels declined rapidly in the first week of BMS-788329 monotherapy but then rose again sharply during the second week (**Figure 3a,b**). A second mutation, NS5A Y93C, emerged and replaced the wild-type strain during the initial 2 weeks of BMS-788329 monotherapy in both mice (**Figure 3a,b** and **Supplementary Tables 3** and **4** online). HCV RNA levels declined to undetectable levels in one of the two mice (**Figure 3a**). In the other mouse, viral breakthrough occurred during combination therapy with the two drugs (**Figure 3b**). The frequency of the NS3-resistant V36A strain increased to 97.8% during the course of combination therapy (**Figure 3b**, **Supplementary Table 4** online). These results indicate that the NS5A L31V strain may rapidly accumulate an additional V36A mutation. Such strains may easily develop resistance against telaprevir, as well.

Effect of BMS-788329 in combination with telaprevir or NS5B inhibitor in mice infected with clones with multiple drug-resistant mutations

We also established a HCV genotype 1b KT-9 clone with both L31V and Y93H mutations in the NS5A region in a chimeric mouse. The mouse was treated with BMS-788329 alone for the first 2 weeks to confirm resistance and then treated with telaprevir plus BMS-788329 combination therapy for 14 weeks. At that point (week 24), telaprevir was replaced with NS5B inhibitor in combination with BMS-788329 for a further 5 weeks (**Figure 4a**). HCV RNA levels in this mouse showed poor response to BMS-788329 alone. Furthermore, HCV RNA rebounded during combination treatment with BMS-788329 and telaprevir. A drug-resistant NS3-V36A strain predominated at weeks 12 and 14, and by weeks 16 and 17 an NS3 T54A strain had emerged (**Figure 4a** and **Supplementary Table 5** online). When we withdrew telaprevir and treated the mice with a combination of BMS-788329 and NS5B inhibitor, HCV RNA rapidly declined and became undetectable. However, the virus rebounded almost immediately and rapidly increased to almost 10^6 copies/ml (**Figure 4a**). Sequencing of the virus detected a resistant NS5B P495S strain (**Figure 4a** and **Supplementary Table 5** online). At week 29, direct sequencing indicated a mixture of wild-type and mutant strains at NS3 aa36 and 54 (data not shown).

Finally, we established an infection in a chimeric mouse with a HCV genotype 1b KT-9 clone with triple resistance mutations (NS3 V36A, NS5A L31V, and NS5A Y93H). The mouse was treated with BMS-788329 plus telaprevir combination therapy for 2 weeks, followed by combination therapy with BMS-788329 and NS5B inhibitor (**Figure 4b**). As expected, HCV RNA did not decrease during the initial BMS-788329 and telaprevir combination therapy. In contrast, HCV RNA levels declined rapidly during BMS-788329 and NS5B inhibitor combination therapy. HCV RNA remained negative until 11 weeks after cessation of the therapy, after which it increased gradually to nearly pre-treatment levels. Sequence analysis of the virus revealed four resistance mutations: NS3 V36A, NS5A L31V, NS5A Y93H, and NS5B P495S (**Figure 4b,c**). This indicates that mutant strains resistant against all recently developed DAAs might emerge following inappropriate use of drugs and that sequential use of these DAA should be avoided.

DISCUSSION

Although the approval of telaprevir and boceprevir has improved the eradication rate of HCV in patients treated with triple therapy (2–9), the therapy is approved only for genotype 1. Furthermore, severe side effects such as anemia, neutropenia, thrombocytopenia, and appetite loss limit patient eligibility to young and relatively healthy individuals without advanced liver diseases. Unexpected development of severe skin disease results in premature termination of the therapy. Therapies without interferon and ribavirin such as DAA combination therapies (20–22) may provide more tolerable therapy for older patients as well as those with cirrhosis.

We assessed the effect of combination of BMS-788329 plus telaprevir or BMS-821095 using human hepatocyte chimeric mice. We chose these combinations because daclatasvir, which is a close analog of BMS-788329, shows potent antiviral effects with few side effects (30). Furthermore, when we performed a clinical trial of the combination of daclatasvir and asunaprevir, some patients had elevated transaminases and hyperbilirubinemia, probably due to the side effects of asunaprevir (22). We thus attempted to find out a better dual combination of DAAs. Although the combination of telaprevir and BMS-788329 effectively reduced serum virus levels in mice infected with genotype 1b serum, we observed minimal change in HCV RNA levels in mice infected with genotype 2 (32). These results are consistent

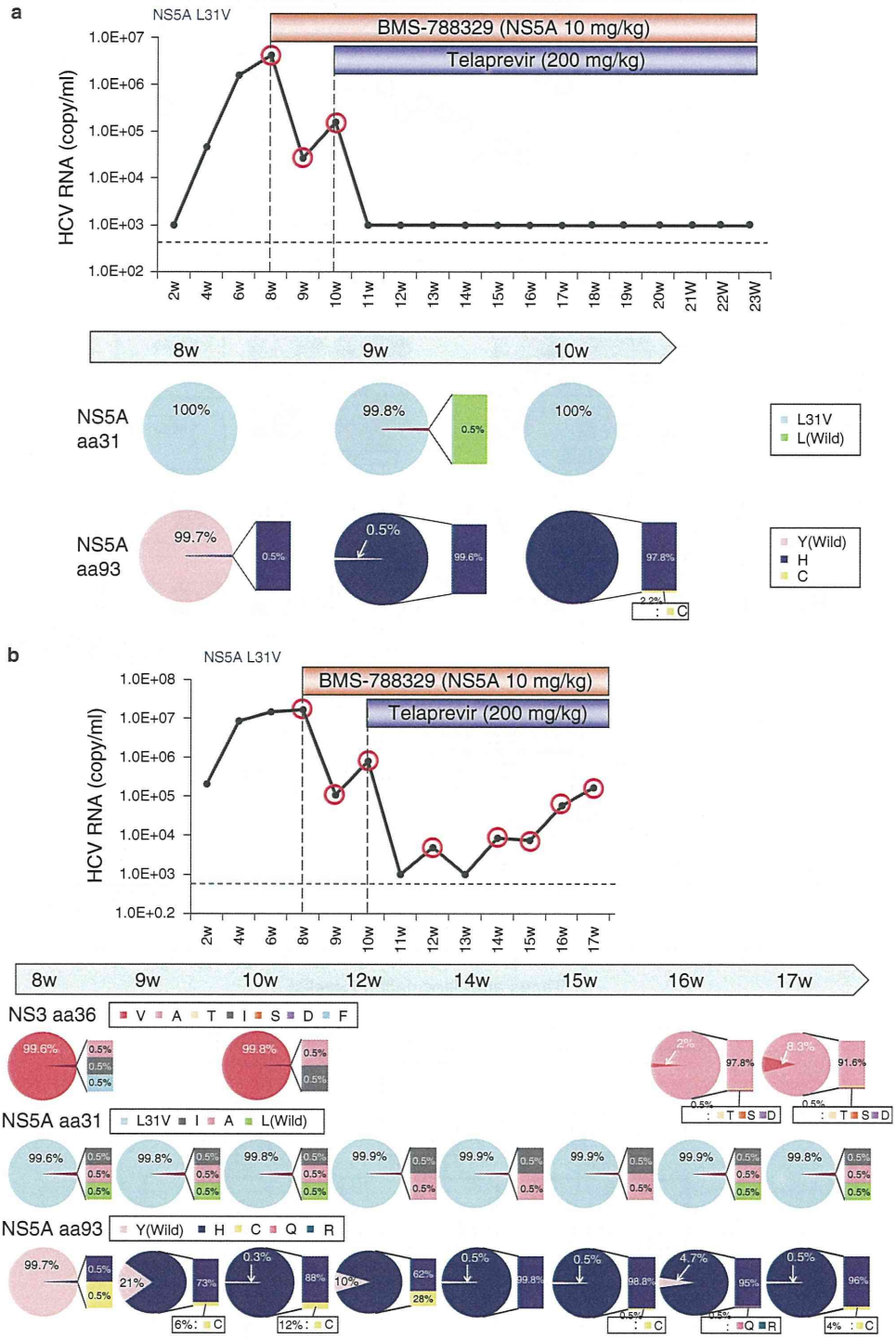

Figure 3. Effect of telaprevir and BMS-788329 combination therapy in human hepatocyte chimeric mice infected with an HCV clone containing NS5A L31V resistance mutation. We infected mice with an infectious clone harboring an NS5A inhibitor-resistant NS5A L31V mutation. (**a**, **b**) Mice received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). HCV, hepatitis C virus; w, weeks.

with *in vitro* experiments showing higher ED50 levels of these drugs against genotype 2 (30,31). The combination of BMS-788329 and telaprevir thus might be a good candidate for clinical trial in patients with genotype 1b infection. Higher dosage or a next-generation NS3 protease inhibitor should be considered for treatment of patients infected with genotype 2.

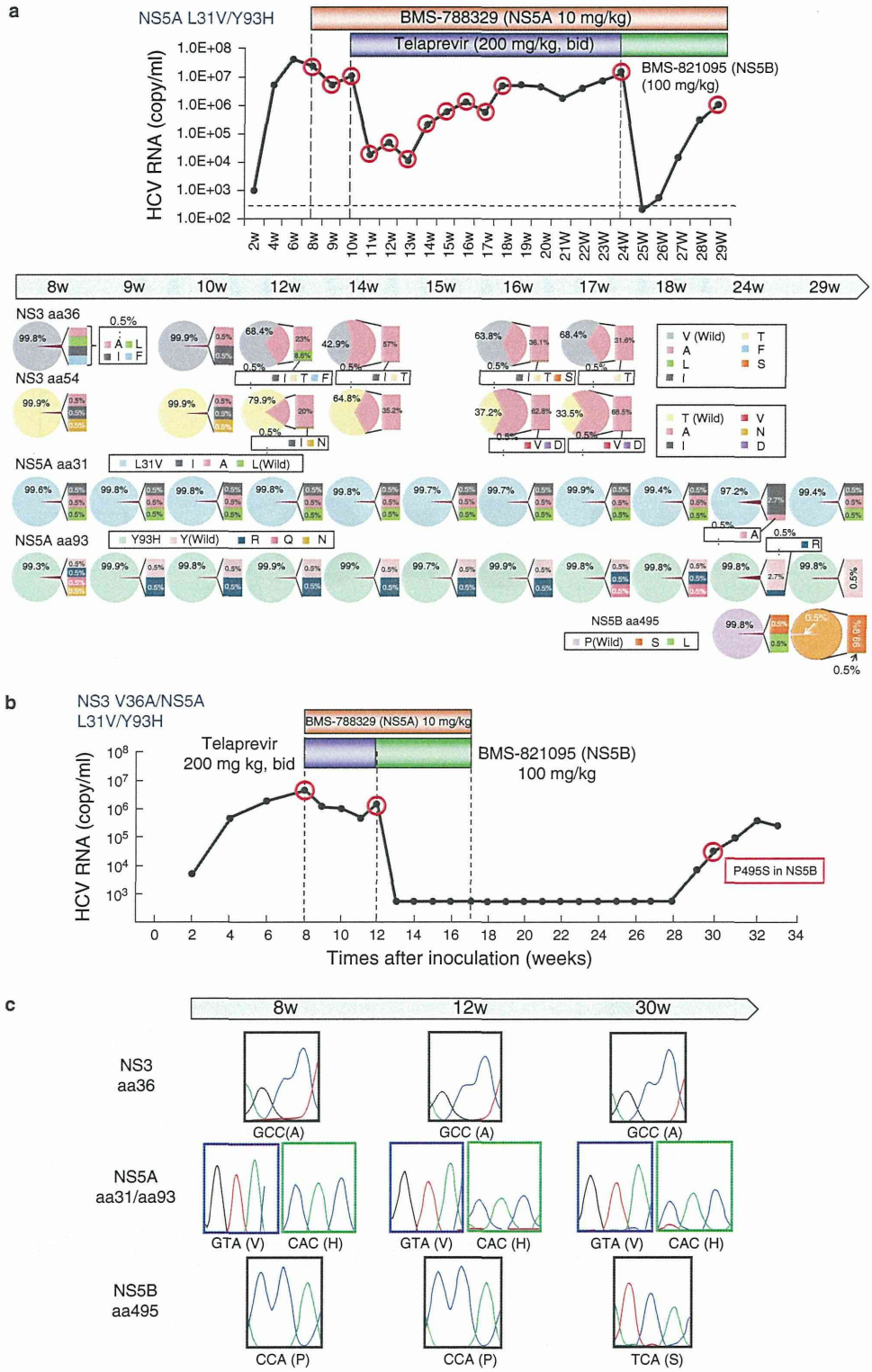

Figure 4. Effect of BMS-788329 in combination with telaprevir or NS5B inhibitor in mice infected with clones with multiple drug-resistant mutations. (**a**) We infected a mouse with an infectious clone harboring NS5A inhibitor-resistant NS5A L31V and Y93H mutations. The mouse received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor). After 14 weeks of telaprevir plus BMS-788329 combination therapy, we replaced telaprevir with 100 mg/kg (mouse body weight) of NS5B inhibitor and continued combination therapy with BMS-788329 for an additional 5 weeks. (**b**, **c**) We infected a mouse with an infectious clone harboring resistance mutations against both telaprevir (NS3 V36A) and NS5A inhibitor (NS5A L31V and Y93H). The mouse received 200 mg/kg (mouse body weight) of telaprevir and 10 mg/kg (mouse body weight) of BMS-788329 (NS5A inhibitor) for 2 weeks, followed by combination therapy with BMS-788329 and 100 mg/kg (mouse body weight) of NS5B inhibitor. W, weeks.

As one of the mice treated with the combination of BMS-788329 and telaprevir showed poor response followed by relapse, we decided to analyze the effect of pre-existing resistance mutations on response to therapy. We infected mice with HCV clones having introduced mutations known to be associated with resistance to specific DAAs. Combination therapy with BMS-788329 and telaprevir effectively suppressed replication of HCV BMS-788329-resistant NS3 V36A HCV (**Figure 2**). This combination might be useful for patients who have a naturally occurring drug resistance profile. In contrast, two mice with a BMS-788329-resistant NS5A L31V mutation easily acquired an additional Y93C mutation, which has been reported to confer very strong resistance against the drug (**Figure 3**) (28). These factors should be considered when we establish future DAA combination therapies.

When we treated a mouse infected with a NS5A L31V and Y93H double mutation with BMS-788329 and telaprevir, the mice rapidly developed resistance against telaprevir. Furthermore, we observed the rapid emergence of an NS5B P495S mutant during combination therapy with BMS-788329 and BMS-821095 (NS5B inhibitor) (**Figure 4**). Such mutant strains with triple resistance features were also observed when the virus reappeared after cessation of a similar treatment (**Figure 4**). These results imply that mutant strains resistant to all three drugs can emerge after sequential use of these DAAs.

DAA combination therapy without interferon and ribavirin is expected to become a primary treatment option in the near future. As we showed in this study, however, multidrug-resistant strains may appear after incomplete, sequential use of DAAs (**Figure 4**). Although simultaneous use of three drugs is the strongest therapy against HCV, side effects related to drug interactions may occur. Therefore, we should further examine possible combinations of DAAs to establish the best combination therapy to eradicate HCV from all treated patients without incurring serious side effect.

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CONFLICT OF INTEREST

Guarantor of the article: Kazuaki Chayama, MD, PhD.

Specific author contributions: H. Abe, N. Hiraga, and M. Imamura designed and performed the experiments. C. N. Hayes analyzed the data. H. Abe and C. N. Hayes wrote the manuscript. M. Tsuge, D. Miki, S Takahashi, and H. Ochi participated in data analysis and discussion. K. Chayama initiated and directed the entire study, designed experiments and wrote the manuscript.

Financial support: None.

Potential competing interests: K.C. is a speaker for BMS, MSD and Roche.

LIVER

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ DAAs against HCV have recently been developed.
- ✓ DAAs have been recommended to be used as interferon-based regimen.
- ✓ DAAs without interferon must be used in combination because of development of resistant strain.
- ✓ Development of multidrug-resistant strains remains to be characterized.

WHAT IS NEW HERE

- ✓ Resistant strains easily develop from cloned virus strains after sequential DAAs combination therapy.
- ✓ Sequential use of DAAs must be avoided to prevent a development of resistant strains.

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