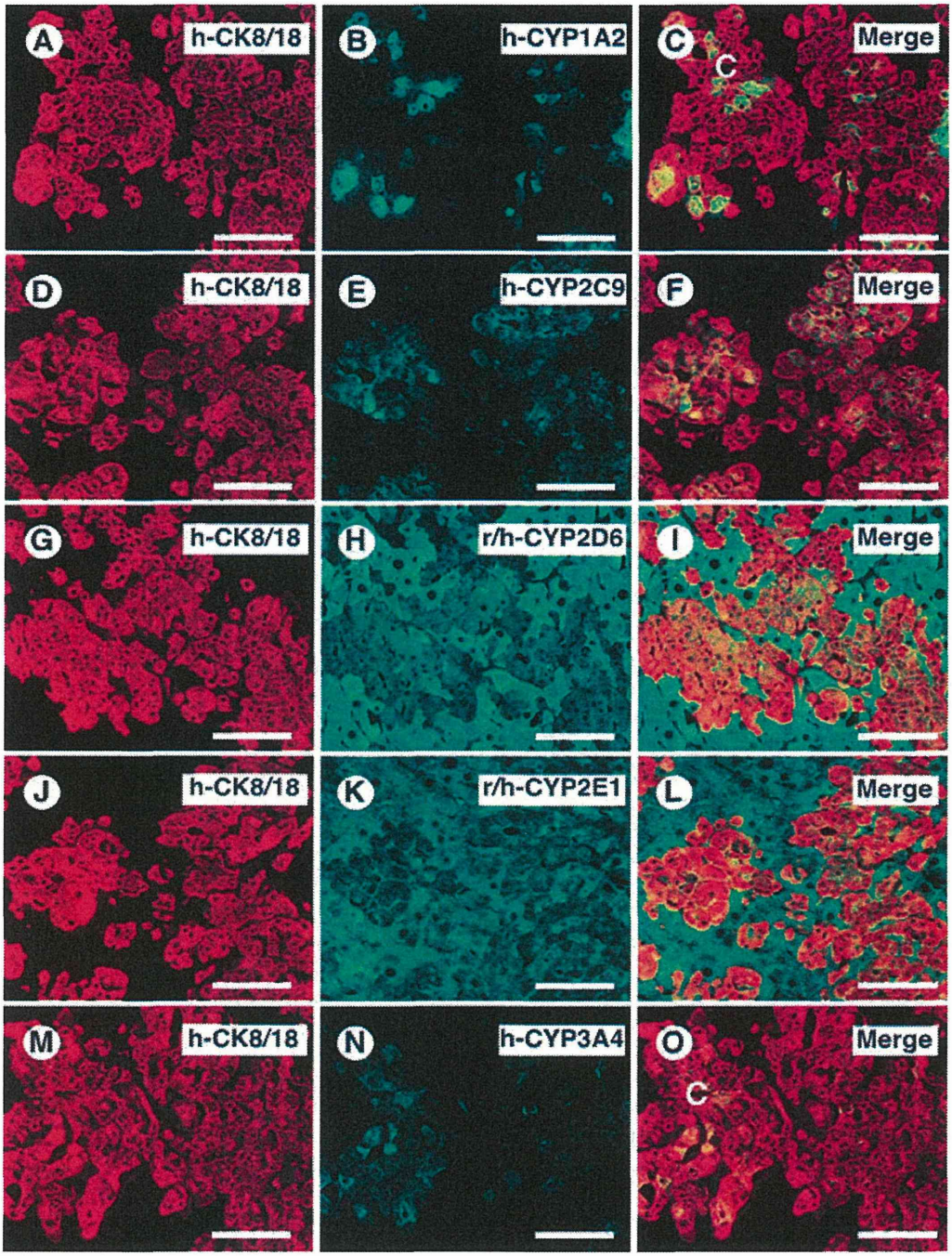

Fig. 5. h-CYP expressions of h-hepatocytes in RS-treated rat livers. Histological sections were prepared from chimeric rat livers at 3 weeks post-transplantation as in Fig. 4B–J. The liver sections were all stained for h-CK8/18 (A, D, G, J, M), which were then for h-CYP1A2 (B), h-CYP2C9 (E), r/h-CYP2D6 (H), r/h-CYP2E1 (K), and h-CYP3A4 (N). H-CYP1A2, 2C9, and 3A4 antibodies were h-specific, and CYP2D6 and 2E1 were not h-specific (r/h). The photographs in the right column (C, F, I, L, O) are obtained by merging the photographs in the left (A, D, G, J, M) and middle columns (B, E, H, K, N) on the corresponding lines. Most h-CK8/18$^+$ cells were also CYP2C9$^+$, 2D6$^+$ and 2E1$^+$. H-CK8/18$^-$ cells near the central veins ("C" in photographs C and O) were positive to CYP1A2 and 3A4. Open bars indicate 100 μm.

Although we cannot make a direct comparison of the proliferative microenvironment of the host liver from these independent studies, our results indicate a distinct similarity between the proliferative microenvironments of the host liver in adults and infants. To compare the pro-proliferative

potential of the adult and infant hepatic microenvironment, we calculated the average size of colonies formed by adult rat donor hepatocytes at 3 weeks after transplantation in adult and infant rat models. The average size of a colony is measured in terms of area in the adult RS/PH rat model and the infant RS rat model and was found to be approximately 26 000 μm^2 [28] and 60 000 μm^2 (this study), respectively. This result indicates that the pro-proliferative potential is higher in the infant liver than in its adult counterpart. This model has some inherent advantages such as PH, quite invasive operation to the host, is unnecessary and the amount of transplanted hepatocytes is reducible quite much in comparison with the RS-/PH-treated adult rat model [18,19]. Our RS-treated infant rat model might be useful as host for liver repopulation studies using a limited number of liver stem cells or progenitor cells derived from adult tissues such as induced pluripotent and embryonic stem cells as donors.

To incorporate xenogeneic hepatocytes into this RS-treated infant rat model, we used FK506 to suppress immune reactivity. We did not notice lymphocyte infiltrations in the FK506-treated chimeric rat livers, which led us to conclude that the FK506 treatment successfully inhibited the host rejection reactions. H-hepatocytes engrafted and repopulated the rat liver at an appreciable level of RI (1 to 5% at 3 week post-transplantation), although the level was much lower than that (11 to 24% at the corresponding time point post-transplantation) accomplished in the transplantation of syngeneic r-hepatocytes. However, the RI value by h-hepatocytes (in the highest case, 5% at 3 weeks post-transplantation, corresponding to 1.4×10^5 ng/ml h-Alb in plasma) was quite high in rat liver compared with the previous study [17,27]. These repopulated h-hepatocytes were concluded to be morphologically normal from microscopical observations on histological sections and also biochemically functional because they expressed the representative h-hepatocyte-specific transcripts and proteins. However, there are also some differences between h-hepatocytes in the chimeric rat liver and in the human body liver. For example, h-CYP1A2, 3A4, and 2E1 expressions were reported to show zonation in normal livers in that only hepatocytes located near the central veins express these proteins [29]. In the present study, the former two CYPs were localized near the central veins, but h-CYP2E1 was expressed diffusedly in liver lobules.

The observed difference in RIs between h- and r-hepatocyte transplantation is not likely to have been caused by the immunosuppressive therapy because the RI of r-hepatocytes did not change in the host rats that received FK506 daily. Wu et al. [30] reported that FK506 perturbed neither transplanted cell engraftment nor proliferation in syngeneic r-hepatocyte transplantation using the RS/PH model, which supports our results. Recently, we generated chimeric mice using uPA/SCID mice as hosts and concordant (rat) and disconcordant (human) hepatocytes as donors [25,31]. The h-hepatocytes proliferated more slowly in the mouse liver and required longer periods of time to achieve near-complete repopulation than the r-hepatocytes, suggesting the difference in proliferation kinetics of h- and r-hepatocytes. This difference could explain the lower RIs of h-hepatocytes. In addition, r- and h-hepatocytes might respond differently to the hepatic microenvironments in the host liver. Molecular incompatibilities in a discordant xenogeneic combination (human to mouse) may greatly impair the engraftment and/or growth efficiency compared with those in a concordant xenogeneic combination (rat to mouse) [24,25].

This study was performed by comparing the results and data on the chimeric mouse model. We transplanted 1.0×10^6 human child (6 yrs old) hepatocytes/6 g body weight (1.67×10^5 cells/g) in the mouse model [24]. However, in the rat model, we transplanted the same donor cells, but in lower quantities (5.0×10^5 cells/20 g body weight, 0.25×10^5 cells/g), accounting for 85% less donor cells/g body weight. The transplant was delivered through the spleen in the mouse model, but through the portal vein in the rat model. Irrespective of the fact that only 15% of donor cells in the mouse model were transplanted, h-Alb levels at 3 weeks post-transplantation (0.4 to 1.4×10^5 ng/ml) in the rat model were comparable to those (1.7 to 5.9×10^5 ng/ml) in the mouse model [24]. When child (9 months old) cells were injected, h-Alb levels at 3 weeks post-transplantation were $2.3 \pm 0.6 \times 10^5$ ng/ml ($n = 3$) ($7.4 \pm 4.1\%$ in the repopulation rate) [25] and $0.9 \pm 0.4 \times 10^5$ ng/ml ($n = 5$) ($2.5 \pm 1.5\%$) for the mouse and rat models, respectively. These data showed that engraftment in the host liver and early growth in the rat model took place at comparable levels to the mouse model. This finding indicates that the number of adopted h-hepatocytes was sufficient for repopulation at comparable levels to the mouse model. As such, no problems exist concerning the quality of the donor cells. We also examined donor cell quality prior to transplantation. Taken together, we concluded that h-hepatocytes are able to engraft the host liver and grow well therein, for at least 3 weeks post-transplantation.

The combined treatment of infant rats with RS and FK506 was lethal, whereas the singular treatment with RS or FK506 did not influence mortality. Although r-hepatocyte transplantation could improve mortality rates at some extent, h-hepatocytes could not. This may be attributable to the above-mentioned differences in growth kinetics between the h- and r-hepatocytes. We are not able to utilize the presently developed humanized rat model for studies that require long-term observations of the xenograft. The depletion of immune cells by antibodies against immune-responsive cells [32–34] could be an alternative method to the administration of immunosuppressive agents. Recently, X-SCID rats were generated using the zinc-finger nuclease method [35]. X-SCID or SCID rats will be preferable hosts for the production of h-hepatocyte chimeric rats. The RS-treated infant rat model presented in this study should be applicable for X-SCID or SCID rats to develop liver-humanized chimeric rats. In the present study, infant rats were treated with 10 mg/kg of RS before hepatocyte transplantation. Rats younger than approximately 20 days are known to be more sensitive to the hepatotoxic effects of RS than older animals. Gender-based differences in the metabolism of RS were significantly observed at approximately 30 days [36]. In our preliminary study, we treated 1- and 2-week-old infant rats with RS. When r-hepatocytes were transplanted into 1-week-old RS-treated rats, the repopulation index at 3 weeks after transplantation was lower than that at 2 weeks after transplantation. We also attempted 2 RS doses—5 mg/kg and 10 mg/kg—because the LD50 of RS is known to be 10 to 14 mg/kg in 2-week-old rats [36]. When 5 mg/kg of RS was injected into 2-week-old rats, we observed that liver damage was not severe and the liver to body weight ratio did not change. Thus, we selected 10 mg/kg as the final dose. Currently, it is considered that the conditions to treat rats with RS for damaging the rat liver should be optimized to obtain a better host survival rate. Overexpression of uPA in the mouse model has been well accepted as a suitable method to induce host liver injury for repopulation by xenogeneic hepatocytes. This is not the case for RS treatment.

Rodents are widely used as animal models for investigating the physiology and pathology of the liver. However, the liver characteristics of different species are well documented, especially between humans and rodents. This hinders the ability to correlate experimental data from animal models to the human. Therefore, h-hepatocytes are vital tools to investigate liver function, and, as such, it is important to know their physiological and pathological characteristics under experimentally controllable conditions. Our ultimate goal is to generate rats with >70% h-hepatocytes. It is evident that these rats (hepatocyte-humanized rats) greatly contribute to deepening our understanding concerning the characteristics of h-hepatocytes. This rat model is superior to the conventional mouse model because blood samples are more easily accessible and available in larger volumes. These samples are a critical source of understanding the characteristics of h-hepatocytes. This rat model will be useful in pharmacokinetic studies, HBV- or HCV-infection studies, toxicity tests, and carcinogenicity studies for drug development. In addition, this model will prove to be an important tool for xenograft studies with the ultimate goal of enhancing our understanding of clinical hepatocyte transplantation across species.

Acknowledgments

We thank Ms. Y. Yoshizane, Ms. S. Nagai, Ms. H. Kohno, and Ms. Y. Matsumoto for their excellent technical assistance, and all members of the CLUSTER and PhoenixBio Co., Ltd., for advice and helpful discussion. We greatly thank Dr. W. H. Chambers (University of Pittsburgh Cancer Institute, Pittsburgh, PA) for providing us with hybridoma-producing 3.2.3 antibodies.

Author contributions

AT and CT conceived, designed, and coordinated the study and wrote the draft manuscript. CT and KY revised the manuscript.

References

1. Nishitai R, Koch CA, Ogata K et al. Toward the survival and function of xenogeneic hepatocyte grafts. Liver Transpl 2005; 11: 39.
2. David P, Alexandre E, Audet M et al. Engraftment and albumin production of intrasplenically transplanted rat hepatocytes (Sprague–Dawley), freshly isolated versus cryopreserved, into Nagase analbuminemic rats (NAR). Cell Transplant 2001; 10: 67.
3. Kato R. Characteristics and differences in the hepatic mixed function oxidases of different species. Pharmacol Ther 1979; 6: 41.
4. Green CE, LeValley SE, Tyson CA. Comparison of amphetamine metabolism using isolated hepatocytes from five species including human. J Pharmacol Exp Ther 1986; 237: 931.
5. Naritomi Y, Terashita S, Kimura S, Suzuki A, Kagayama A, Sugiyama Y. Prediction of human hepatic clearance from in vivo animal experiments and in vitro metabolic studies with liver microsomes from animals and humans. Drug Metab Dispos 2001; 29: 1316.

6. Guha C, Mohan S, Roy-Chowdhury N, Roy-Chowdhury J. Cell culture and animal models of viral hepatitis Part I: hepatitis B. Lab Anim 2004; 33: 37.
7. Durantel D, Zoulim F. Going towards more relevant cell culture models to study the in vitro replication of serum-derived hepatitis C virus and virus/host cell interactions? J Hepatol 2007; 46: 1.
8. Tateno C, Yoshizane Y, Saitou N et al. Near-completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004; 165: 901.
9. Yoshizato K, Tateno C. A human hepatocyte-bearing mouse: an animal model to predict drug metabolism and effectiveness in humans. PPAR Res 2009; 2009: 476217.
10. Yoshizato K, Tateno C. In vivo modeling of human liver for pharmacological study using humanized mouse. Expert Opin Drug Metab Toxicol 2009; 5: 1435.
11. Katoh M, Matsui T, Okamura H et al. Expression of human phase II enzymes in chimeric mice with humanized liver. Drug Metab Dispos 2005; 33: 1333.
12. Nishimura M, Yoshitsugu H, Yokoi T et al. Evaluation of mRNA expression of human drug-metabolizing enzymes and transporters in chimeric mouse with humanized liver. Xenobiotica 2005; 35: 877.
13. Tsuge M, Hiraga N, Takaishi H et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. Hepatology 1046; 2005: 42.
14. Dandri M, Burda MR, Török E et al. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 2001; 33: 981.
15. Suemizu H, Hasegawa M, Kawai K et al. Establishment of a humanized model of liver using NOD/Shi-scid IL2Rg (null) mice. Biochem Biophys Res Commun 2008; 377: 248.
16. Meuleman P, Libbrecht L, de Vos R et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. Hepatology 2005; 41: 847.
17. Igarashi Y, Tateno C, Tanaka Y et al. Engraftment of human hepatocytes in the livers of rats reconstructed with bone marrow cells from an immunodeficient mouse. Xenotransplantation 2008; 15: 235.
18. Laconi E, Oren R, Mukhopadhyay DK et al. Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am J Pathol 1998; 153: 319.
19. Katayama S, Tateno C, Asahara T, Yoshizato K. Size-dependent in vivo growth potential of adult rat hepatocytes. Am J Pathol 2001; 158: 97.
20. Mori M, Mori Y, Hotta D et al. Changes in the size of liver lobules during neonatal growth of rat liver. Kanzo 1982; 23: 575.
21. van Rooijen N, Sanders A. Liposome mediated depletion of macrophages: mechanism of action, preparation of liposomes and applications. J Immunol Methods 1994; 174: 83.
22. van den Brink MR, Hunt LE, Hiserodt JC. In vivo treatment with monoclonal antibody 3.2.3 selectively eliminates natural killer cells in rat. J Exp Med 1990; 171: 197.
23. Tateno C, Takai-Kajihara C, Yamasaki C, Sato H, Yoshizato K. Heterogeneity of growth potential of adult rat hepatocytes in vitro. Hepatology 2000; 31: 65.
24. Masumoto N, Tateno C, Tachibana A et al. GH enhances proliferation of human hepatocytes grafted into immunodeficient mice with damaged liver. J Endocrinol 2007; 194: 529.
25. Utoh R, Tateno C, Kataoka M et al. Hepatic hyperplasia associated with discordant xenogeneic parenchymal–nonparenchymal interactions in human hepatocyte-repopulated mice. Am J Pathol 2010; 177: 654.
26. Wu CH, Ouyang EC, Walton C et al. Human hepatocytes transplanted into genetically immunocompetent rats are susceptible to infection by hepatitis B virus in situ. J Viral Hepat 2001; 8: 111.
27. Ouyang EC, Wu CH, Walton C et al. Transplantation of human hepatocytes into tolerized genetically immunocompetent rats. World J Gastroenterol 2001; 7: 324.
28. Nishie M, Tateno C, Utoh R et al. Hepatocytes from fibrotic liver possess high growth potential in vivo. Cell Transplant 2009; 18: 665.
29. Oinonen T, Lindros KO. Zonation of hepatic cytochrome P-450 expression and regulation. Biochem J 1998; 329: 17.
30. Wu YM, Joseph B, Gupta S. Immunosuppression using the mTOR inhibition mechanism affects replacement of rat liver with transplanted cells. Hepatology 2006; 44: 410.
31. Emoto K, Tateno C, Hino H et al. Efficient in vivo xenogeneic retroviral vector-mediated gene transduction into human hepatocytes. Hum Gene Ther 2005; 16: 1168.
32. Pruitt SK, Baldwin WM III, Bartii RN, Sanfilippo F. The effect of xenoreactive antibody and B cell depletion on hyperacute rejection of guinea pig-to-rat cardiac xenografts. Transplantation 1993; 56: 1318.
33. Soares M, Havaux X, Nisol F et al. Modulation of rat B cell differentiation in vivo by the administration of an anti-mu monoclonal antibody. J Immunol 1996; 156: 108.
34. Geissler I, Sawyer GJ, Dong X et al. The effect of anti-lymphocyte serum on subpopulations of blood and tissue leucocytes: possible supplementary mechanisms for suppression of rejection and the development of opportunistic infections. Transpl Int 2005; 18: 1366.
35. Mashimo T, Takizawa A, Voigt B et al. Generation of knockout rats with X-linked severe combined immunodeficiency (X-SCID) using zinc-finger nucleases. PLoS ONE 2010; 25: e8870.
36. Mattocks AR, White IN. Toxic effects and pyrrolic metabolites in the liver of young rats given the pyrrolizidine alkaloid retrorsine. Chem Biol Interact 1973; 6: 297.

Gut Online First, published on January 15, 2013 as 10.1136/gutjnl-2012-302600

ORIGINAL ARTICLE

Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice

Niu Shi,[1,2] Nobuhiko Hiraga,[1,2] Michio Imamura,[1,2] C Nelson Hayes,[1,2] Yizhou Zhang,[1,2] Keiichi Kosaka,[1,2] Akihito Okazaki,[1,2] Eisuke Murakami,[1,2] Masataka Tsuge,[1,2] Hiromi Abe,[1,2] Hiroshi Aikata,[1,2] Shoichi Takahashi,[1,2] Hidenori Ochi,[2,3] Chise Tateno-Mukaidani,[2,4] Katsutoshi Yoshizato,[2,4] Hirotaka Matsui,[5] Akinori Kanai,[6] Toshiya Inaba,[5] Fiona McPhee,[7] Min Gao,[7] Kazuaki Chayama[1,2,3]

[1]Department of Gastroenterology and Metabolism, Applied Life Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, Hiroshima, Japan
[2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan
[3]Laboratory for Digestive Diseases, Center for Genomic Medicine, The Institute of Physical and Chemical Research (RIKEN), Hiroshima, Japan
[4]PhoenixBio Co., Ltd., Higashihiroshima, Japan
[5]Department of Molecular Oncology and Leukemia Program Project, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan
[6]Radiation Research Center for Frontier Science, Research Institute for Radiation Biology and Medicine, Hiroshima University, Hiroshima, Japan
[7]Bristol-Myers Squibb, Research and Development, Wallingford, Connecticut, USA

Correspondence to
Professor Kazuaki Chayama, Department of Gastroenterology and Metabolism, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan; chayama@mba.ocn.ne.jp

Received 29 March 2012
Revised 18 December 2012
Accepted 20 December 2012

To cite: Shi N, Hiraga N, Imamura M, *et al. Gut* Published Online First: [*Please include* Day Month Year] doi:10.1136/gutjnl-2012-302600

ABSTRACT

Objective We recently demonstrated that combination treatment with NS3 protease and NS5B polymerase inhibitors succeeded in eradicating the virus in genotype 1b hepatitis C virus (HCV)-infected mice. In this study, we investigated the effect of combining an NS5A replication complex inhibitor (RCI) with either NS3 protease or NS5B inhibitors on elimination of HCV genotypes 1b, 2a and 2b.

Design The effects of Bristol-Myers Squibb (BMS)-605339 (NS3 protease inhibitor; PI), BMS-788329 (NS5A RCI) and BMS-821095 (NS5B non-nucleoside analogue inhibitor) on HCV genotypes 1b and 2a were examined using subgenomic HCV replicon cells. HCV genotype 1b, 2a or 2b-infected human hepatocyte chimeric mice were also treated with BMS-605339, BMS-788329 or BMS-821095 alone or in combination with two of the drugs for 4 weeks. Genotypic analysis of viral sequences was achieved by direct and ultra-deep sequencing.

Results Anti-HCV effects of BMS-605339 and BMS-821095 were more potent against genotype 1b than against genotype 2a. In in-vivo experiments, viral breakthrough due to the development of a high prevalence of drug-resistant variants was observed in mice treated with BMS-605339, BMS-788329 and BMS-821095 in monotherapy. In contrast to monotherapy, 4 weeks of combination therapy with the NS5A RCI and either NS3 PI or NS5B inhibitor succeeded in completely eradicating the virus in genotype 1b HCV-infected mice. Conversely, these combination therapies failed to eradicate the virus in mice infected with HCV genotypes 2a or 2b.

Conclusions These oral combination therapies may serve as a Peg-alfa-free treatment for patients chronically infected with HCV genotype 1b.

Significance of this study

What is already known on this subject?
- Anti-HCV drug monotherapy for chronic hepatitis C patients often results in viral breakthrough due to the emergence of drug-resistant clones.
- Combination treatment of NS3 PI and NS5A inhibitor can eradicate genotype 1b HCV in chronic hepatitis C patients without interferon.

What are the new findings?
- Combination treatment of NS5A inhibitor with either NS3 PI or NS5B inhibitor can eradicate HCV, but the effect differs among HCV genotypes.

How might it impact on clinical practice in the foreseeable future?
- Short-term combination of NS5A inhibitor with either NS3 PI or NS5B inhibitor might provide an effective interferon-free treatment for genotype 1b chronic hepatitis C patients; however, the combination treatment might be less effective against genotype 2.

INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseases, such as cirrhosis and hepatocellular carcinoma.[1 2] A number of new selective inhibitors of HCV proteins, termed direct-acting antiviral agents (DAA), are currently under development. HCV inhibitors targeting NS3 protease and NS5A and NS5B polymerase activity have proceeded to clinical trials for HCV-infected patients. DAA are used in combination with Peg-alfa and ribavirin because monotherapy with these drugs results in the early emergence of drug-resistant variants.[3 4] As Peg-alfa/ribavirin treatment is frequently associated with serious adverse events, an oral Peg-alfa/ribavirin-free DAA combination therapy would offer an ideal treatment option for chronic hepatitis C patients. The first proof-of-concept study to combine NS3 protease and NS5B inhibitors (INFORM-1) reported that 13 days of this combination treatment achieved robust antiviral suppression in genotype 1 HCV-infected patients, and no evidence of resistance to either compound was observed.[5] Following the INFORM-1 study, we and other groups have also reported that a DAA-only

combination comprising NS3 protease inhibitor (PI), Bristol-Myers Squibb (BMS)-650032 (asunaprevir) and NS5A replication complex inhibitor (RCI), BMS-790052 (daclatasvir) can achieve high sustained virological response (SVR) rates in patients with HCV genotype 1b infection.[6] A number of DAA-only combination studies are now entering phase 2 clinical trials.[7] The effect of telaprevir was recently analysed in genotype 2 HCV-infected patients. Fifteen days of telaprevir monotherapy decreased the serum HCV RNA titre by 3.7 $\log_{10}$ IU/ml, and 3 months of telaprevir plus 24 weeks of Peg-alfa/ribavirin triple therapy resulted in SVR in 100% of genotype 2 HCV-infected patients.[8] However, the effect of Peg-alfa/ribavirin-free DAA combination therapy on genotype 2 HCV has not been reported.

The immunodeficient urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes that can be infected with HCV.[9] This animal model is useful for evaluating anti-HCV drugs such as Peg-alfa and NS3 PI.[10 11] Using this animal model, we recently described the successful elimination of HCV genotype 1b by treatment with a combination of NS3 protease and NS5B inhibitors.[12] In this study, we investigated whether short-term combination treatments with NS5A RCI and either NS3 protease or NS5B site I inhibitors could eliminate HCV *in vivo* in human hepatocyte chimeric mice, and we compared the efficacy of the drugs against HCV genotype 1 versus genotype 2.

METHODS

Compounds and cells

BMS-605339 (NS3 PI, analogue of asunaprevir), BMS-788329 (NS5A RCI, analogue of daclatasvir) and BMS-821095 (NS5B non-nucleoside analogue inhibitor; NNI) were synthesised by BMS. Huh-7 cells that stably maintain HCV replicons were propagated as subconfluent monolayers in Dulbecco's modified essential medium containing 10% fetal bovine serum, 2 mM L-glutamine, and 0.5 mg/ml geneticin (G418; Invitrogen Corp., Carlsbad, California, USA) at 37°C under 5% carbon dioxide.[13]

Determination of IC_{50} in culture systems

The genotype 1b (Con 1) replicon cell line was constructed as described previously.[14] A genotype 2a (JFH-1) cell line was generated by introducing the JFH-1 sequence from NS3 to NS5B into the genotype 1b (Con 1) backbone.[15] Inhibition of HCV RNA replication by either BMS-605339, BMS-788329 or BMS-821095 for 72 h was monitored using a luciferase reporter assay. Antiviral activities of the compounds, for example, the 50% inhibitory concentration (IC_{50}), were determined as described previously.[16]

Human serum samples

Human serum containing a high titre of HCV genotypes 1b, 2a and 2b was obtained from patients with chronic hepatitis who had given written informed consent to participate in the study. Serum samples were divided into small aliquots and stored in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee.

Animal treatment

Generation of the $uPA^{+/+}/SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously.[17] All mice were transplanted with frozen human hepatocytes obtained from the same donor. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples and killing of animals were performed under ether anaesthesia. Eight weeks after hepatocyte transplantation, mice were injected intravenously with 100 µl of HCV-positive human serum samples. Mice serum samples were obtained every 1 or 2 weeks after HCV infection, and HCV RNA levels were measured.

Treatment of HCV-infected mice with anti-HCV inhibitors

Eight weeks after HCV infection when the mice developed stable viraemia (6–8 $\log_{10}$ copies/ml), mice were administered orally with one of the following: 75 mg/kg of BMS-605339 (twice a day); 10 or 30 mg/kg of BMS-788329 (once a day); or 30 or 100 mg/kg of BMS-821095 (once a day) for 4 weeks. To analyse the effect of the combination treatment, BMS-788329 was mixed with either BMS-605339 or BMS-821095 and given together as a cocktail. To analyse susceptibility to Peg-alfa, 10 µg/kg of human Peg-alfa (Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) were administered by intramuscular injection twice a week for weeks.

RNA extraction and amplification

RNA extraction, nested PCR and quantitation of HCV by real-time PCR were performed as described previously.[11 12] Briefly, RNA was extracted from serum samples and extracted livers using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV complementary DNA was performed using a light cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limit of real-time PCR is 3 $\log_{10}$ copies/ml.

Sequence analysis

The nucleotide and amino acid sequences of the NS3, NS5A and NS5B regions of HCV were determined by direct sequencing as described previously.[12] The primers used to amplify the NS3 region were 5′–GTGCTCCAAGCTGGCATAAC–3′ and 5′–AGGACCGAGGAATCGAACAT–3′ as the first (outer) primer pair and 5′–CTAGAGTGCCGTACTTCGTG–3′ and 5′–ACT GATCCTGGAGGCGTAGC–3′ as the second (inner) primer pair. The primers used to amplify the NS5A region were 5′–GAA TGCAGCTCGCCGAGCAA–3′ and 5′–CCATGTTGTGGTGGC GCAGC–3′ as the first (outer) primer pair and 5′–GCAGCTGT TGGCAGCATAGGTC–3′ and 5′–GATGGTAGTGCATGTCG CC–3′ as the second (inner) primer pair. The primers used to amplify the NS5B region were 5′–TAAGCGAGGAGGCT GGTGAG–3′ and 5′–CCTATTGGCCTGGAGTGTTT–3′ as the first (outer) primer pair and 5′–GACTCAACGGTCAC TGAGAG–3′ and 5′–CCTATTGGCCTGGAGTGTTT–3′ as the second (inner) primer pair. The amplified DNA fragments were separated onto a 2% agarose gel and purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems Inc., California, USA). The obtained nucleotide and amino acid sequences were compared with the prototype sequences of genotype 1b HCV-J (GenBank accession no.: D90208)[18].

Ultra-deep sequencing

We have adapted multiplex sequencing by synthesis to sequence multiple genomes simultaneously using the Illumina genome analyser. Briefly, cDNA was fragmented using sonication, and the resultant fragment distribution was assessed using the Agilent BioAnalyzer 2100 platform. A library was prepared

Table 1 In-vitro activity of BMS-605339, BMS-788329 and BMS-821095 in HCV replicon assays

	IC_{50} (nM)		
Genotype (strain)	BMS-605339	BMS-788329	BMS-821095
1b (Con 1)	3.5±0.8	0.012±0.005	3.8±0.6
2a (JFH-1)	81±27	0.014±0.007	365±266

Data are represented as means±SD from at least three independent experiments.
HCV, hepatitis C virus.##

using the Multiplexing sample preparation kit (Illumina Inc., California, USA). Imaging analysis and base calling were performed using Illumina Pipeline software with default settings. The N-terminal 1344 nucleotides of NS3 protease, 1146 nucleotides of NS5A RCI and 1133 nucleotides of NS5B polymerase were analysed. This technique revealed an average coverage depth of over 1000 sequence reads per base pair in the unique regions of the genome. Read mapping to a reference sequence was performed using BWA.[19] Direct sequencing consensus data were used to improve alignment to the reference sequence. Codon counts were merged and analysed using R V.2.14.

Statistical analysis

Mice serum HCV RNA titres were compared using the Mann–Whitney U test. A p value less than 0.05 was considered statistically significant.

RESULTS

Antiviral activities of BMS-605339, BMS-788329 and BMS-821095 in cell culture systems

The inhibitory effects of BMS-605339, BMS-788329 and BMS-821095 on HCV replication were analysed *in vitro* using HCV replicon cells (genotype 1b, Con 1 and genotype 2a, JFH1). A summary of the IC_{50} values for each drug is shown in table 1. Antiviral activities of BMS-605339 and BMS-788329 were similar to asunaprevir[15] and daclatasvir,[20] respectively. BMS-605339 and BMS-821095 IC_{50} values were 23-fold and 116-fold more potent against genotype 1b than against genotype 2a, respectively.

Peg-alfa treatment on mice infected with HCV genotypes 1 and 2

We first analysed the effect of Peg-alfa on mice infected with HCV genotypes 1 and 2. Mice were injected with 10^5 copies of HCV obtained from patients infected with HCV genotypes 1b, 2a, or 2b. Administration of 10 μg/kg of human Peg-alfa twice a week for 2 weeks resulted in only a 0.53 $\log_{10}$ decrease in the serum HCV RNA titre in HCV genotype 1b-infected mice (figure 1). In contrast, the same therapy resulted in 1.9 $\log_{10}$ and 1.5 $\log_{10}$ decreases in serum HCV RNA titres in mice with HCV genotypes 2a ($p<0.05$) and 2b (not significant), respectively. No decline in HCV RNA titre was observed in control mice infected with HCV genotype 1b during this 2-week period (figure 1). These results are consistent with the clinical observation that genotype 2 demonstrates a higher susceptibility to Peg-alfa treatment compared to HCV genotype 1.

Effects of BMS-605339, BMS-788329, or BMS-821095 on HCV genotype 1b in mice

We analysed the effect of DAA monotherapy on mice infected with HCV genotype 1b. Nine mice were injected with 10^5 copies

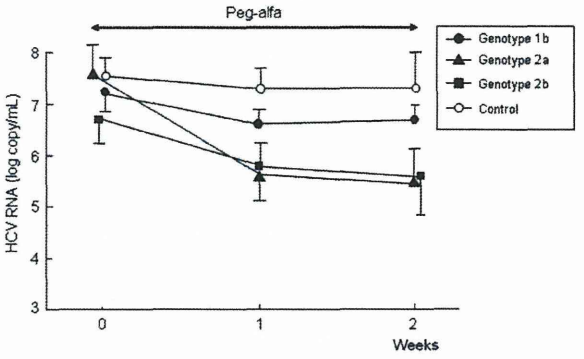

Figure 1 Antiviral effects of Peg-alfa treatment in mice. Mice were infected with hepatitis C virus (HCV) genotypes 1b (n=3), 2a (n=4) or 2b (n=4), then treated with 10 μg/kg of Peg-alfa twice per week for 2 weeks. HCV-infected mice without treatment (n=3) were also analysed (control). Mice serum HCV RNA titres were measured at the indicated times. Data are presented as mean±SD.

of HCV obtained from a patient infected with genotype 1b. Eight weeks after injection when stable viraemia had developed, mice were treated with BMS-605339 (N3PI) (figure 2A), BMS-788329 (NS5A RCI) (figure 2B) or BMS-821095 (NS5B site I inhibitor) (figure 2C) for 4 weeks. Although all BMS-605339 and BMS-788329-treated mice showed an initial reduction of serum HCV RNA titres, all later showed rebound during treatment. Nucleotide analysis by direct sequencing revealed the emergence of a mutation coding for D168E in the NS3 region (NS3 PI-resistant variant)[21] in a BMS-605339-treated mouse (figure 2A), and a mutation coding for Y93H in the NS5A region (NS5A RCI-resistant variant)[14] in a BMS-788329-treated mouse at week 4 of treatment (figure 2B). Almost all mice treated with BMS-821095 showed an initial reduction in serum HCV RNA titres, and also showed rebound with the emergence of mutations coding for P495A and P495S in the NS5B region (NS5B site I inhibitor-resistant variant)[22] at week 4 of treatment (figure 2C). HCV RNA titre reduction was not obvious in some mice treated with 30 mg/kg of BMS-821095 (figure 2C), suggesting that exposure of this inhibitor at 30 mg/kg dosing was not sufficient to suppress viral replication. Ultra-deep sequence analysis showed the development of a high prevalence of drug-resistant variants in mice sera in the NS3 PI, NS5A RCI-treated mice, and enrichment of pre-existing resistance variants in the NS5B NNI-treated mouse 4 weeks after the beginning of the treatment (figure 2D).

Combination treatment of BMS-788329 with either BMS-605339 or BMS-821095 in HCV genotype 1b mice

As monotherapies with either the NS3 PI, or the NS5A RCI or the NS5B NNI were unable to eradicate HCV RNA due to the emergent resistance variants, we analysed the effects of combining the NS5A RCI with either the NS3 PI or NS5B NNI. Mice infected with HCV genotype 1b (two mice per combination group) were treated with 10 mg/kg of BMS-788329 and either 75 mg/kg twice daily of BMS-605339 or 100 mg/kg of BMS-821095 for 4 weeks. In all mice, HCV RNA became negative by nested PCR 1 week after the beginning of combination therapy and remained undetectable after cessation of treatment (figure 3A,B). Elimination of the virus was assumed as HCV RNA was undetectable by nested PCR in mice livers treated with BMS-788329 and either BMS-605339 or BMS-821095 8 weeks

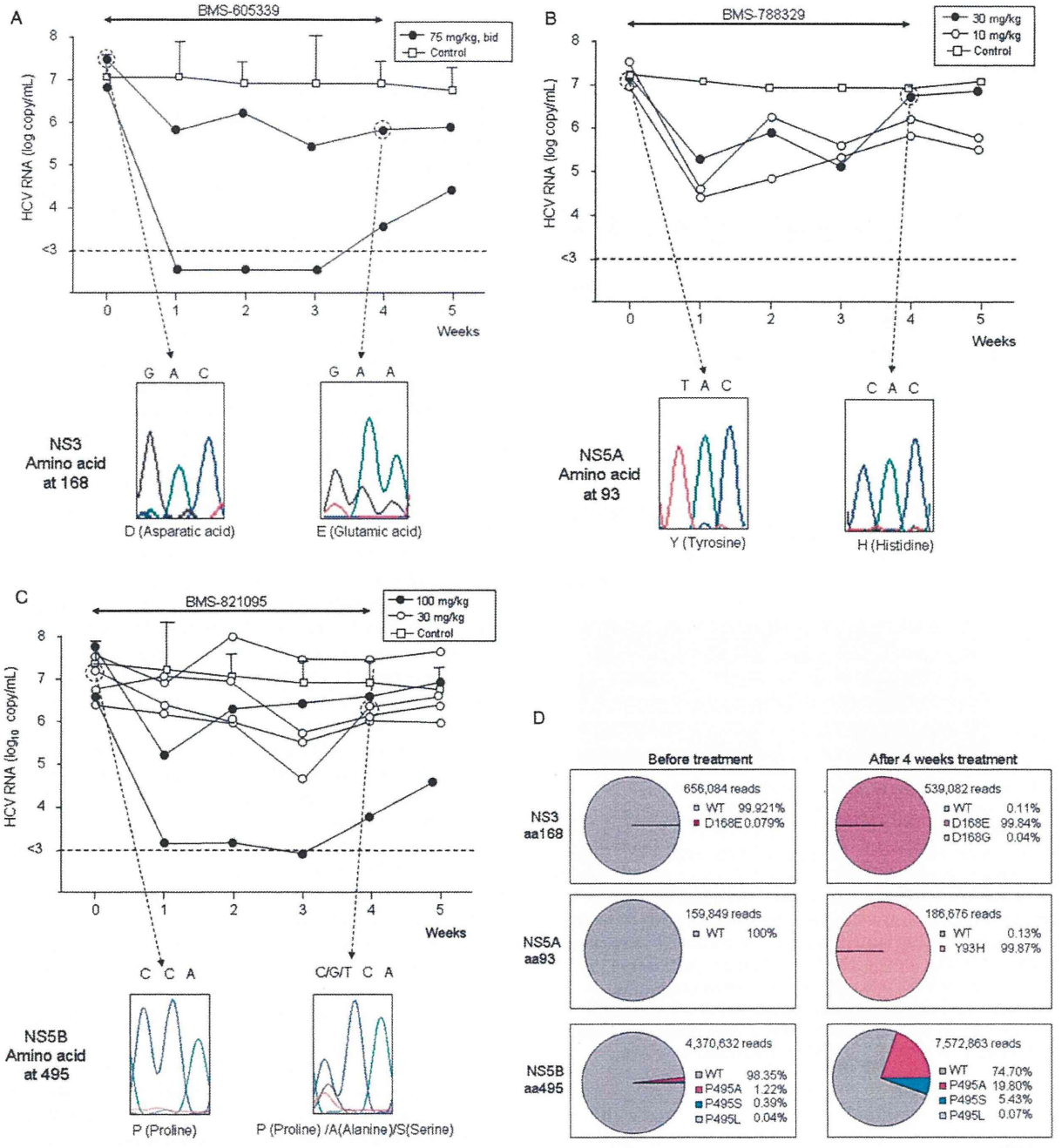

Figure 2 Antiviral effects of BMS-605339, BMS-788329 or BMS-821095 monotherapy in mice infected with hepatitis C virus (HCV) genotype 1b. Mice were injected intravenously with 10^5 copies of HCV genotype 1b. Eight weeks after HCV infection, mice were treated with the indicated concentrations of BMS-605339 (A), BMS-788329 (B) or BMS-821095 (C) for 4 weeks. Serum samples were obtained at the indicated times, and HCV RNA titre and nucleotide and amino acid (aa) sequences were analysed. HCV-infected mice without treatment were also analysed (control). The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml). (D) The aa frequencies in the BMS-605339 (top), BMS-788329 (middle bottom) or BMS-821095 (bottom) treated mice by ultra-deep sequencing before treatment and at 4 weeks are shown.

(week 12) and 7 weeks (week 11) after cessation of therapy, respectively (figure 3C).

Combination treatment of BMS-788329 with either BMS-605339 or BMS-821095 in HCV genotype 2 mice

We analysed the effect of DAA combination therapies on mice infected with HCV genotypes 2a and 2b. In contrast to mice with genotype 1b, mice with genotypes 2a or 2b failed to respond to 4 weeks of treatment with BMS-788329 and BMS-605339 (figure 4A,B). Although the combination of BMS-788329 with BMS-821095 revealed no detectable viral load decline at the time points examined in genotype 2a mice, viral load reductions were detected in genotype 2b mice. Sequence analysis revealed no emergence of resistance variants in the NS3, NS5A or NS5B regions before and 4 weeks after the end of each of these combination treatments, suggesting insufficient drug selection pressure (data not shown).

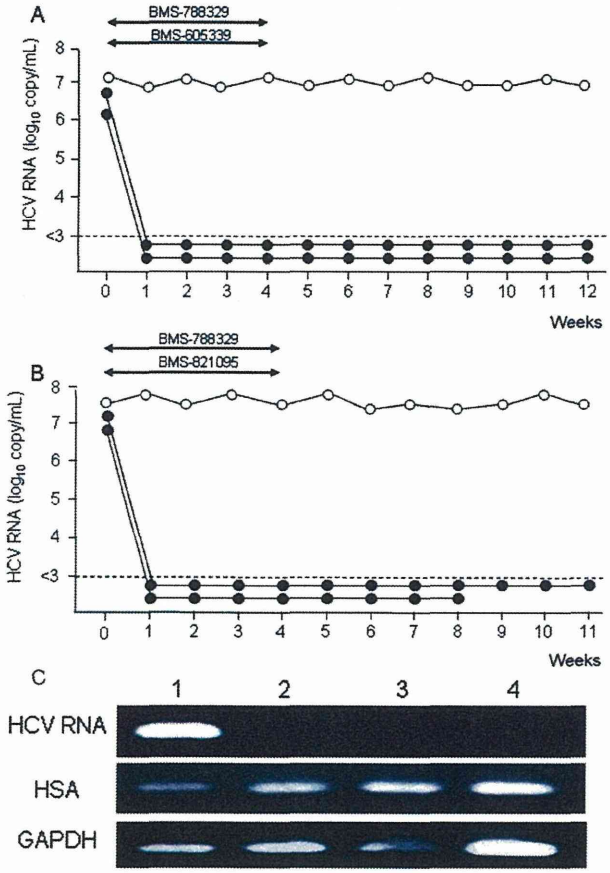

Figure 3 Antiviral effects of NS5A replication complex inhibitor combinations with either an NS3 protease inhibitor or an NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 1b. The four mice were treated with 10 mg/kg of BMS-788329 and either 75 mg/kg twice daily of BMS-605339 (A) or 100 mg/kg of BMS-821095 (B) for 4 weeks (closed circles). Mice without treatment were also analysed (open circles). Serum samples were obtained at the indicated times, and HCV RNA titres were measured. The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml). (C) Nested PCR of HCV RNA, human serum albumin and GAPDH in mouse livers. Livers from mice treated with BMS-788329 and either BMS-605339 (lane 2) or BMS-821095 (lane 3) were obtained. Mouse livers with (lane 1) or without (lane 4) HCV infection were also analysed.

DISCUSSION

DAA-only therapy may offer a promising option to eradicate HCV without incurring the severe side effects of Peg-alfa. However, the emergence of drug-resistant variants is expected for all DAA[21] and has already been observed in combination therapies with two DAA.[5 23 24] If the exposure of the drugs can be safely increased, as we recently reported for a two-drug combination administered to human hepatocyte chimeric mice,[12] eradication of virus is still possible. In this study, we tested the ability of different two-DAA combination therapies to eradicate HCV. Although DAA monotherapies resulted in a viral breakthrough due to the development of a high prevalence of drug-resistant variants (figure 2A–D), DAA combination therapies with the NS5A RCI and either the NS3 PI or NS5B NNI were shown to eradicate virus successfully from HCV genotype 1b-infected mice with only 4 weeks of treatment (figure 3). These two-DAA combination treatments resulted in more rapid, robust declines within the first week of treatment when compared with the suboptimal antiviral responses from each of their respective monotherapies. Furthermore, regimens containing NS5A RCI appeared equally effective in treating mice chronically infected with hepatitis C genotype 1b.

In contrast to the rapid decrease in HCV RNA in mice infected with HCV genotype 1b, HCV genotype 2a and 2b-infected mice either did not respond or responded poorly to treatment with the NS5A RCI combined with either the NS3 PI or NS5B NNI (figure 4A,B). In this study, NS3 PI and NS5B NNI IC_{50} values against genotype 1b were markedly more potent than against genotype 2a in cell culture systems (table 1). These findings are consistent with previous experimental results that reported reduced activity of these drug classes against genotype 2.[25–28] In clinical trials, telaprevir monotherapy was found to result in a rapid decrease in serum HCV RNA levels in patients infected with HCV genotype 2; however, another protease inhibitor, BILN-2061, was less effective in patients with HCV genotype 2 compared to genotype 1.[29] Sequence analysis revealed a pre-existing A156G variant in the NS3 region, a L31M variant in the NS5A region and a I482L variant in the NS5B region in both HCV genotypes 2a and 2b infecting strains used in this study (data not shown). These NS3-A156G and NS5A-L31M variants confer resistance to inhibitors with similar chemical structures to BMS-605339 and BMS-788329, respectively, in genotype 2a replicon cell culture assays.[30–32] Although BMS-788329 was very potent against the genotype-2a JFH-1 replicon (IC_{50} 0.014 nM; table 1), its activity was significantly less against other genotype 2a and 2b viruses, such as genotype 2a HC-J6CF. The loss in potency observed in these viruses is not surprising because these viruses have a methionine at NS5A amino acid residue 31. The IC_{50} of a genotype 2a hybrid replicon containing HC-J6CF NS5A with L31M substitution is approximately 10 nM (data not shown). The minimal antiviral response in mice infected with genotypes 2a and 2b receiving treatments containing BMS-788329 with either BMS-605339 or BMS-821095 can therefore be explained by pre-existing NS3, NS5A and NS5B resistance variants. Nevertheless, it is possible that mice infected with wild-type genotype 2 viruses and subsequently treated with higher doses of each of these DAA in dual or even triple combination therapy may have resulted in more robust reductions in viral load. The human hepatocyte chimeric mouse model offers a viable approach for identifying effective DAA-only combinations that not only act against HCV genotype 1 but against all HCV genotypes.

In summary, we demonstrated that an NS5A RCI can be effectively combined with different inhibitor classes to cure human hepatocyte chimeric mice infected with HCV genotype 1b after 4 weeks of treatment. However, these treatment combinations were not effective against HCV genotype 2. Oral combinations incorporating an NS5A RCI might offer Peg-alfa-free treatment options for genotype 1b chronic hepatitis C patients.

Acknowledgements The authors would like thank Rie Akiyama and Yoko Matsumoto for their expert technical help, and Bristol-Meyers Squibb Research and Development for providing BMS-605339, BMS-788329 and BMS-821095 and suggesting the experimental design.

Funding This study was supported in part by a grant-in-aid for scientific research from the Japanese Ministry of Labour, Health and Welfare.

Competing interests MG and FM are employees of Bristol-Myers Squibb. All other authors declare no competing interests.

Ethics approval The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved a priori by the institutional review committee. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care.

Patient consent Obtained.

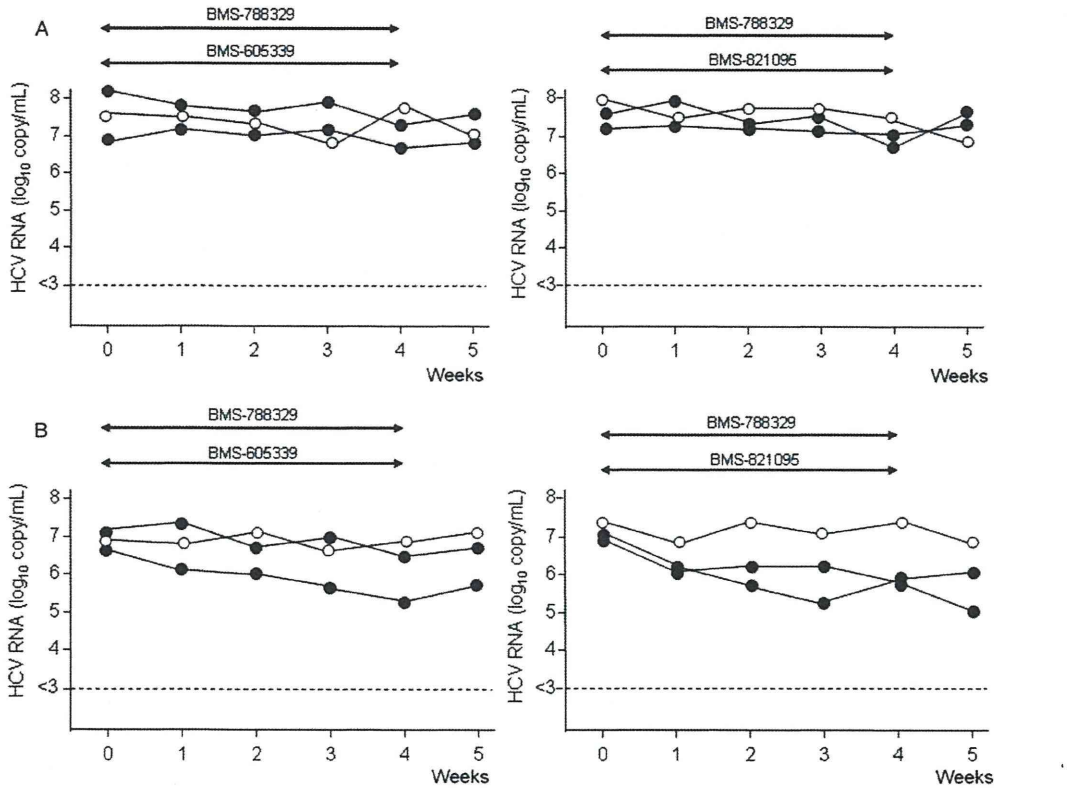

Figure 4 Antiviral effects of NS5A replication complex inhibitor combinations with either NS3 protease inhibitor or NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 2. Each of the four HCV genotype 2a (A) or 2b (B) infected mice were treated with 10 mg/kg of BMS-788329 combined with either 75 mg/kg twice daily of BMS-605339 (left panel) or 100 mg/kg of BMS-821095 (right panel) for 4 weeks (closed circles). Mice without treatment were also analysed (open circles). Serum samples were obtained at the indicated times, and HCV RNA titres were measured. The horizontal dotted line indicates the HCV RNA titre detection limit (3 log copies/ml).

Provenance and review Not commissioned; externally peer reviewed.

REFERENCES

1 Kiyosawa K, Sodeyama T, Tanaka E, *et al*. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990;12:671–5.
2 Niederau C, Lange S, Heintges T, *et al*. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. *Hepatology* 1998;28:1687–95.
3 Lin C, Gates CA, Rao BG, *et al*. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. *J Biol Chem* 2005;280:36784–91.
4 Mo H, Lu L, Pilot-Matias T, *et al*. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor in vitro. *Antimicrob Agents Chemother* 2005;49:4305–14.
5 Gane EJ, Roberts SK, Stedman CA, *et al*. Oral combination therapy with a nucleoside polymerase inhibitor (RG7128) and danoprevir for chronic hepatitis C genotype 1 infection (INFORM-1): a randomised, double-blind, placebo-controlled, dose-escalation trial. *Lancet* 2010;376:1467–75.
6 Chayama K, Takahashi S, Toyota J, *et al*. Dual therapy with the nonstructural protein 5A inhibitor, daclatasvir, and the nonstructural protein 3 protease inhibitor, asunaprevir, in hepatitis C virus genotype 1b-infected null responders. *Hepatology* 2012;55:742–8.
7 Gane E. Future hepatitis C virus treatment: interferon-sparing combinations. *Liver Int* 2011;31(Suppl. 1):62–7.
8 Foster GR, Hezode C, Bronowicki JP, *et al*. Telaprevir alone or with peginterferon and ribavirin reduces HCV RNA in patients with chronic genotype 2 but not genotype 3 infections. *Gastroenterology* 2011;141:881–9; e881.
9 Mercer DF, Schiller DE, Elliott JF, *et al*. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;7:927–33.
10 Kneteman NM, Weiner AJ, O'Connell J, *et al*. Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* 2006;43:1346–53.
11 Hiraga N, Imamura M, Tsuge M, *et al*. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett* 2007;581:1983–7.
12 Ohara E, Hiraga N, Imamura M, *et al*. Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice. *J Hepatol* 2011;54:872–8.
13 Don RH, Cox PT, Wainwright BJ, *et al*. 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* 1991;19:4008.
14 Fridell RA, Qiu D, Wang C, *et al*. Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an in vitro replicon system. *Antimicrob Agents Chemother* 2010;54:3641–50.
15 McPhee F, Sheaffer AK, Friborg J, *et al*. Preclinical profile and characterization of the hepatitis C virus NS3 protease inhibitor asunaprevir (BMS-650032). *Antimicrob Agents Chemother* 2012;56:5387–96.
16 Sheaffer AK, Lee MS, Hernandez D, *et al*. Development of a chimeric replicon system for phenotypic analysis of NS3 protease sequences from HCV clinical isolates. *Antiviral Ther* 2011;16:705–18.
17 Tateno C, Yoshizane Y, Saito N, *et al*. Near completely humanised liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004;165:901–12.
18 Kato T, Matsumura T, Heller T, *et al*. Production of infectious hepatitis C virus of various genotypes in cell cultures. *J Virol* 2007;81:4405–11.
19 Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 2010;26:589–95.
20 Gao M, Nettles RE, Belema M, *et al*. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* 2010;465:96–100.
21 Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* 2010;138:447–62.
22 Kukolj G, McGibbon GA, McKercher G, *et al*. Binding site characterization and resistance to a class of non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase. *J Biol Chem* 2005;280:39260–7.
23 McHutchison JG, Everson GT, Gordon SC, *et al*. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009;360:1827–38.
24 Hezode C, Forestier N, Dusheiko G, *et al*. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009;360:1839–50.