ける。また、本研究で使用するヒト由来試料はすでに樹立された細胞株であり倫理面での問題はないと考えられるが、新たにヒト組織などを使用する必然性が生じた場合には、文部科学省等でまとめられた「ヒトゲノム、遺伝子解析研究に関する倫理指針」及び、平成 13 年 3 月 29 日付 12 文科振第 266 号文部科学省研究振興局長通知に則り、当該研究機関の医学研究倫理審査委員会に申請し、インフォームドコンセントに係る手続きを実施し、提供試料、個人情報を厳格に管理保存する。

C. 研究結果

遺伝子型2bのHCV株のレプリコンは野生型、S2208I、A2217S の変異挿入ではコロニー形成が見られなかったが、LSG 変異（F438L、A15S、D559G）の挿入によりコロニー形成が観察された。合計 15 クローンのレプリコン細胞を樹立してレプリコンゲノムの配列を決定した。すべてのレプリコン細胞においてLSG変異以外の新たな適合変異が検出された。適合変異はNS4B, NS5A, NS5B 領域に認められた。検出された 8 種類の適合変異を遺伝子型2b株の全長遺伝子にLSG変異とともに導入してウイルス産生実験をおこなった。全長構築から試験管内でRNAを合成して、Huh751 細胞にトランスフェクションし、RNA 導入細胞を経代培養した。その結果感染性ウイルスの産生が確認された。

D. 考察

遺伝子型2b株のレプリコンゲノムに検出した適合変異を利用することにより、感染性ウイルスを作成することが可能となった。遺伝子型2bは我が国では1b, 2aについで感染が広がっているため、抗ウイルス薬の感受性や、耐性ウイルスの研究が必要である。今後、この実験系を用いて、臨床で使用されている抗ウイルス薬や開発中の薬剤に対する解析が可能となる。

E. 結論

遺伝子型2bの感染性HCVを作成することができた。

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G.知的所有権の出願・登録状況

なし

厚生労働省科学研究費補助金（肝炎等克服緊急対策研究事業）
創薬と新規治療法開発に資するヒト肝細胞キメラマウスを用いた肝炎ウイルス制御に関する研究
分担報告書（平成25年度）

ヒト肝細胞キメラマウスを用いた新規抗HCV薬の効果判定

研究分担者　今村道雄　広島大学病院消化器・代謝内科　診療講師

研究要旨：uPA/SCID マウスにヒト肝細胞を移植したヒト肝細胞キメラマウスを用いてリバースジェネティクスの手法により，野生型あるいは direct-acting antiviral agent（DAA）耐性変異型 C 型肝炎ウイルス（HCV）感染マウスを作製した．Telaprevir あるいは NS5A 阻害剤耐性型 HCV 感染マウス，に telaprevir+NS5A 阻害剤を併用投与すると両薬剤に対する 2 重耐性型 HCV が出現し breakthrough が生じ，さらに NS5B 阻害剤の投与により，3 重耐性型 HCV が出現した．DAA 製剤を sequential に使用すると，多剤耐性変異型 HCV が出現するため，注意が必要であることが示された．
uPA/SCIDマウスよりさらに免疫不全であるNOGマウスにHerpes simplex virus type 1 thymidine kinase（HSVtk）遺伝子を過剰発現させたTK-NOGマウスを用いてヒト肝細胞キメラマウスを作製しHCVを感染させた．TK-NOG マウスはuPA/SCID マウスに比べヒト肝細胞置換率が低値であってもHCVの感染率が高く，HCV 研究に有用な新規のヒト肝細胞キメラマウスに有用になると思われた．

A. 研究目的

C 型肝炎ウイルス(HCV)感染ヒト肝細胞キメラマウスを用いて direct-acting antiviral agent (DAA) 耐性変異型 HCV の出現あるいは genotype 間の治療効果を検討する．さらに uPA/SCID よりさらに免疫不全でるNOGマウスを用いて新規ヒト肝細胞キメラマウスを作製する．

B. 研究方法

Genotype 1b HCV感染性クローンKT9のNS3領域にtelaprevir耐性V36A，NS5A領域にNS5A阻害剤耐性L31V，Y93Hを挿入したクローンの全長cDNAを用いて*in vitro transcription*法によりHCV RNAを合成し，50 μgのRNAをヒト肝細胞移植uPA-SCIDマウスの肝臓内に直接注入し感染マウスを作製後，種々のDAAを投与した．また超免疫不全であるNOGマウスにHerpes simplex virus type 1 thymidine kinase（HSVtk）遺伝子を過剰発現させたTK-NOGマウスに6 mg/kgのガンシクロビル（GCV）を隔日で2回投与しマウス肝細胞のアポトーシスを惹起した．GCV投与1週後にヒト肝細胞を経脾臓的に移植し8週後，HCV陽性ヒト血清を静脈内注射し感染を惹起し，HCV感染率，IFNの薬効を評価した．

C. 結果

Genotype 1b 型 C 型肝炎患者血清を投与し感染させたマウスに telaprevirwを投与したところ耐性変異である NS3 V36A 変異が出現した．また野生型 HCV クローン感染マウスに対し，telaprevir を投与したところ，やはり NS3 V36A 変異が出現し，HCV クローンからも耐性変異が出現することが見出された．Telaprevir 耐性である NS3 V36A 変異クローンを感染させたマウスに対し，telaprevir+NS5A 阻害剤を併用投与したところ，血中 HCV RNA は低下するものの陰性化は得られず，NS3 変異に加え NS5A 阻害剤耐性変異である NS5A Y93H 変異が出現した．

NS5A 阻害剤耐性である NS5A L31V 変異あるいは L31V+Y93H 変異型クローン感染マウスに対し，telaprevir+NS5A 阻害剤を併用投与したところ NS5A の変異に加え NS3 V36A 変異が出現し 2 重耐性型となり breakthrough が生じた.

HCV 感染では，置換率が低値あったマウスにおいても，TK-NOG マウスは uPA-SCID マウスよりも高い割合で感染が成立した．抗ウイルス薬の薬効を評価したところ uPA-SCID マウスと同程度であった.

TK-NOG マウスへの GCV 投与 1 週後の ALT 値が高いほどヒト肝細胞移植 8 週後の血中ヒトアルブミン値（ヒト肝細胞置換率）は高値であった．HCV 感染は高置換率マウス（70%以上）では TK-NOG（10/10 頭）および uPA-SCID マウス（50/53 頭）で同程度であったが，低置換率マウス（70%未満）では uPA-SCID（1/5 頭）に比べ TK-NOG マウス（27/28 頭）において有意に高率であった. 感染成立後のマウス血中 HCV RNA 量，IFN 投与による血中ウイルス低下量は TK-NOG および uPA-SCID マウスにおいてほぼ同程度であった.

D. 考察

DAA 製剤を sequential に使用すると，多剤耐性変異型 HCV が出現するため，注意が必要であることが示された. 新規に作製された TK-NOG マウスを用いたヒト肝細胞は，肝炎ウイルス研究に有用な動物モデルである.

E. 結論

HCV 感染ヒト肝細胞キメラマウスを用いて DAA 耐性ウイルスの検討が可能であった. TK-NOG マウスを用いて HCV 感染が可能なキメラマウスを作製した.

F.健康危機情報

特になし

G．研究発表

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H．知的財産権の出願・登録状況

特になし

Ⅲ．　研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Shi N, Hiraga N, Imamura M, Hayes CN, Zhang Y, Kosaka K, Okazaki A, Murakami E, Tsuge M, Abe H, Aikata H, Takahashi S, Ochi H,	Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice.	Gut	62	1055-61	2013
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Ⅳ.　研究成果の刊行物・別刷

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ORIGINAL ARTICLE

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

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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′–ACTG ATCCTGGAGGCGTAGC–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′–GCAGCT GTTGGCAGCATAGGTC–3′ and 5′–GATGGTAGTGCATGTCG CC–3′ as the second (inner) primer pair. The primers used to amplify the NS5B region were 5′–TAAGCGAGGAGGCTGG TGAG–3′ and 5′–CCTATTGGCCTGGAGTGTTT–3′ as the first (outer) primer pair and 5′–GACTCAACGGTCACTGAGAG–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 using the Multiplexing sample preparation kit (Illumina Inc., California, USA). Imaging analysis and base

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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-788329and 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 withHCV 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-821095on 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 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).

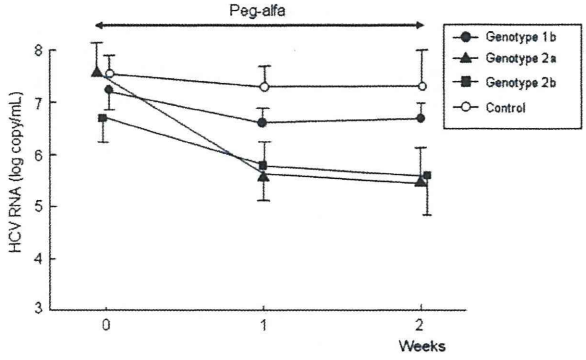

Figure 1 Antiviral effects of Peg-alfa treatment in mice. Mice were infected with hepatitis C virus (HCV) genotypes 1b (n=3), 2a (n=4) or 2b (n=4), then treated with 10 μg/kg of Peg-alfa twice per week for 2 weeks. HCV-infected mice without treatment (n=3) were also analysed (control). Mice serum HCV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

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

Genotype (strain)	IC_{50} (nM) 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.##

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