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G. 知的所有権の取得状況

特になし

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
分担研究報告書（平成24年度）

In vitro, in vivo増殖系を用いたC型肝炎ウイルス増殖のメカニズムの解析と創薬への応用

研究分担者　脇田　隆字　国立感染症研究所・ウイルス第二部　部長

研究要旨：本研究ではC型肝炎ウイルス（HCV）のin vitroおよびin vivo増殖系を用いたウイルス増殖メカニズムの解析をおこない、その結果を創薬へ応用することを目的とする。そのために、新たなウイルス培養増殖系の確立を目指す。

A. 研究目的

C型肝炎ウイルス（HCV）新規感染者は激減したものの、その原因ウイルスであるHCVのキャリアは約１００～２００万人がいまだに存在すると推定されている。その多くが肝硬変から肝臓癌へ移行する。HCV感染に対する治療法はインターフェロンとリバビリンの併用療法が行われているが、その有効率は40-50%程度である。昨年新たな抗ウイルス薬としてプロテアーゼ阻害薬が承認され、治療の有効率は向上すると考えられるが、さらなる治療薬の開発が必要である。肝炎ウイルスに対する新たな治療法の開発は患者の予後を改善するのみならず、肝硬変および肝臓癌という高度な医療が必要な疾患の患者数を減らすことにより、結果的に医療費の低減に寄与し、社会の福祉に寄与することが可能である。HCVのin vitroおよびin vivo増殖系を用いたウイルス増殖メカニズムの解析をおこない、その結果を創薬へ応用することを目的とする。そのために、新たなウイルス培養増殖系の確立を目指す。

B. 研究方法

１．遺伝子型キメラHCV感染実験

遺伝子型1bのCon1株のNS3プロテアーゼ領域およびNS5b領域をそれぞれ遺伝子型2aのJFH-1株に組換えたキメラレプリコンおよびウイルス構築を作成した。プラスミドからウイルスRNAを合成してHuh7細胞またはHuh7.5.1細胞にトランスフェクションし、レプリコン増殖およびウイルスの産生を観察した。

（倫理面への配慮）

各種研究材料の取り扱い及び組換えDNA実験は、適切な申請を行い承認を受ける。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 1b の NS3 を遺伝子型 2a の JFH-1 株に組換えたレプリコン構築（pSGR_JFH_N3PCon1）および NS5b を組換えた構築（pSGR_JFH_N5bSLCon1）は Huh7 細胞で増殖可能であったが、その複製効率は JFH-1 株よりも低く、適合変異が必要であった。レプリコン実験で同定した適合変異を導入した全長キメラウイルス構築（pJFH_N3PCon1 および pJFH_N5bSLCon1）を作成した。全長ウイルス RNA を Huh7.5.1 細胞に導入したところ pJFH_N3PCon1 はウイルスゲノム複製は観察されたが感染性ウイルス粒子産生は見られなかった。pJFH_N5bSLCon1 は複製増殖が可能で感染性ウイルス粒子も産生された。

全長キメラウイルス構築を用いて抗ウイルス薬の効果を観察すると、NS3 プロテアーゼ領域を Con1 に組換えるとプロテアーゼ阻害剤の感受性が高くなった。また NS5b 領域を組換えると遺伝子型 1b と同様の感受性を示した。したがって、これらのキメラウイルス構築は遺伝子型 1b のプロテアーゼ阻害剤およびポリメラーゼ阻害剤の感受性の検討に適していると考えられた。

D. 考察

遺伝子型1bのHCVを培養細胞で増やすことは難しいが、新たなキメラウイルス構築はプロテアーゼ阻害剤やポリメラーゼ阻害剤の検討に適している。薬剤耐性変異の解析などを実施する予定である。

E. 結論

新規キメラウイルス構築の培養細胞における複製増殖、薬剤感受性を検討した。

F. 研究発表

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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
分担研究報告書（平成24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　助教

研究要旨：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．NS5A阻害剤+第二世代protease阻害剤あるいはNS5A阻害剤+非核酸型ポリメラーゼ阻害剤の併用療法はgenotype 1b型HCVには有効であるが，genotype 2aまたは2b型HCVには有効性は低かった．その原因として，2aまたは2b型HCVにはすでにこれら薬剤に対する耐性変異を有している症例が存在することを見出し，そのような症例では効果が弱い可能性が示された．

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．

B．研究方法

ヒト肝細胞キメラマウスにgenotype 1b型のHCV全長クローンから合成したRNAを肝臓内に注入し感染させ，種々のDAA製剤を投与し治療効果および耐性ウイルスの出現を検討した．野生型HCVクローンにDAA耐性変異を挿入したRNAを作製し，耐性変異型HCV感染マウスも作製した．またgenotype 1b，2a，2bの患者血清を感染させたマウスにNS5A阻害剤+プロテアーゼ阻害剤あるいはNS5A阻害剤＋非核酸型ポリメラーゼ阻害剤の併用療法を4週間行い，ウイルス変異と治療効果の関係を検討した．

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．

NS5A阻害剤+第二世代protease阻害剤あるいはNS5A阻害剤+非核酸型NS5B阻害剤の併用療法

により，genotype 1b 型 HCV 感染マウスからのマウス血中 HCV RNA は開始1週後には陰性化し，4 週間の投与中，再上昇を認めなかった．治療終了後も血中ウイルス陰性化は継続し，HCV は排除されたものと思われた．一方，genotype 2a あるいは 2b 型感染マウスでは，血中 HCV RNA はほとんど低下しなかった．これらマウスの HCV を direct sequence にて検討したところ，治療前，genotype 2a 型では，NS3 A156G，NS5A L31M，NS5B I482L および V484A 変異，genotype 2b 型では NS3 A156G および D168A，NS5A L31M，NS5B I482L，V484A および V499A と第2世代プロテアーゼ阻害剤，NS5A 阻害剤，非核酸型ポリメラーゼ阻害剤の耐性変異を有していた．

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．NS5A 阻害剤+第二世代 protease 阻害剤あるいは NS5A 阻害剤+非核酸型ポリメラーゼ阻害剤の併用療法は genotype 1b 型 HCV には有効であるが，genotype 2a または 2b 型 HCV はこれら薬剤に対する耐性変異を有している症例が存在し，そのような症例では効果が弱い可能性が示された．

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．

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

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
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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′–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

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.##

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

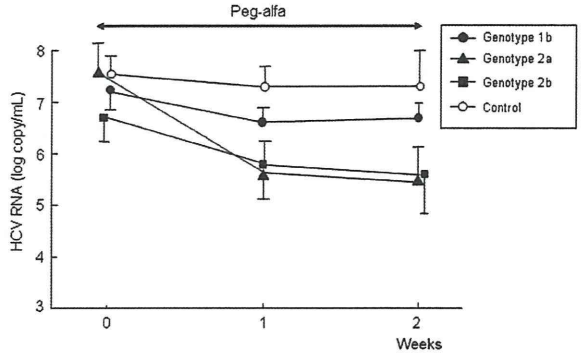

Figure 1 Antiviral effects of Peg-alfa treatment in mice. Mice were infected with hepatitis C virus (HCV) genotypes 1b (n=3), 2a (n=4) or 2b (n=4), then treated with 10 μg/kg of Peg-alfa twice per week for 2 weeks. HCV-infected mice without treatment (n=3) were also analysed (control). Mice serum HCV 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

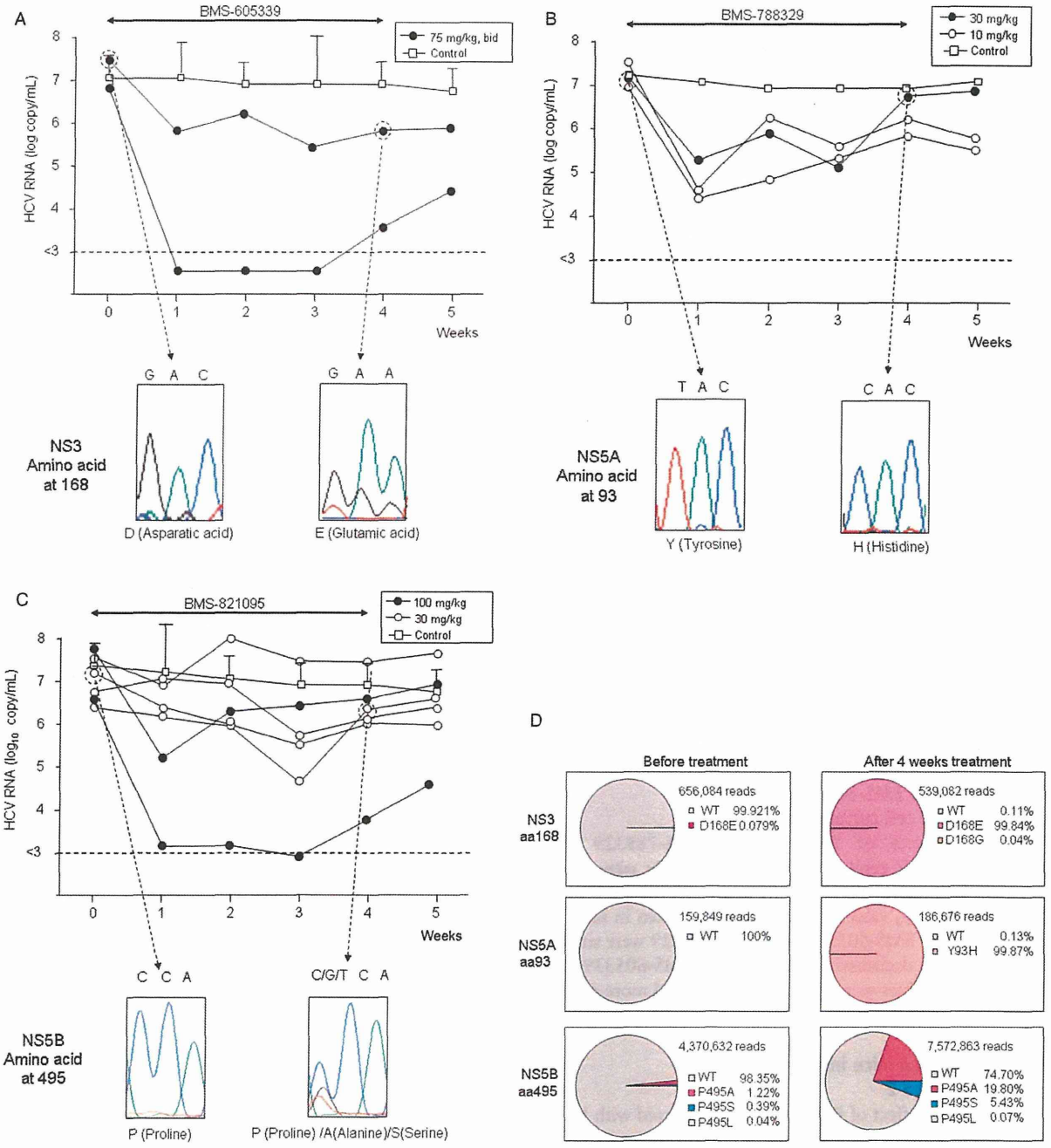

Figure 2 Antiviral effects of BMS-605339, BMS-788329 or BMS-821095 monotherapy in mice infected with hepatitis C virus (HCV) genotype 1b. Mice were injected intravenously with 10^5 copies of HCV genotype 1b. Eight weeks after HCV infection, mice were treated with the indicated concentrations of BMS-605339 (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).

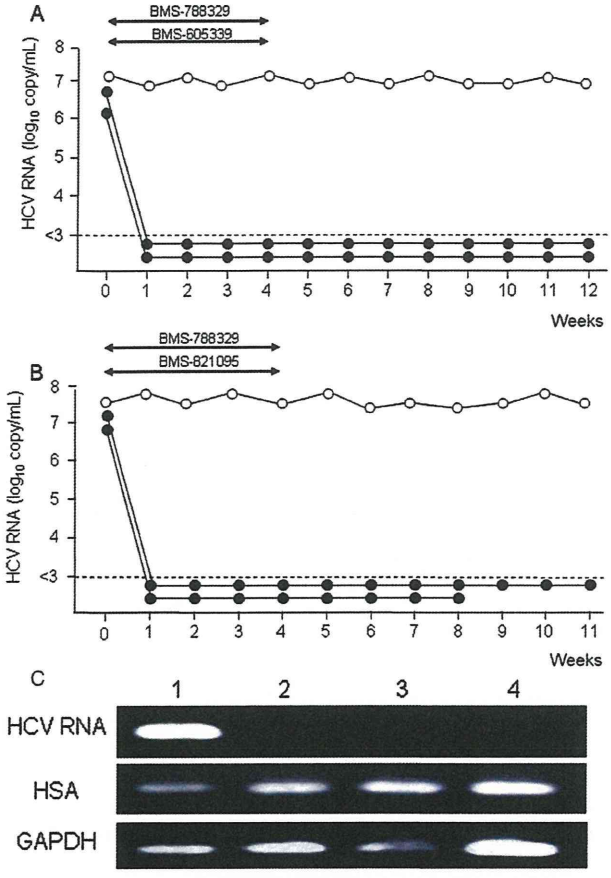

Figure 3 Antiviral effects of NS5A replication complex inhibitor combinations with either an NS3 protease inhibitor or an NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 1b. The four mice were treated with 10 mg/kg of BMS-788329 and either 75 mg/kg twice daily of BMS-605339 (A) 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.

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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.

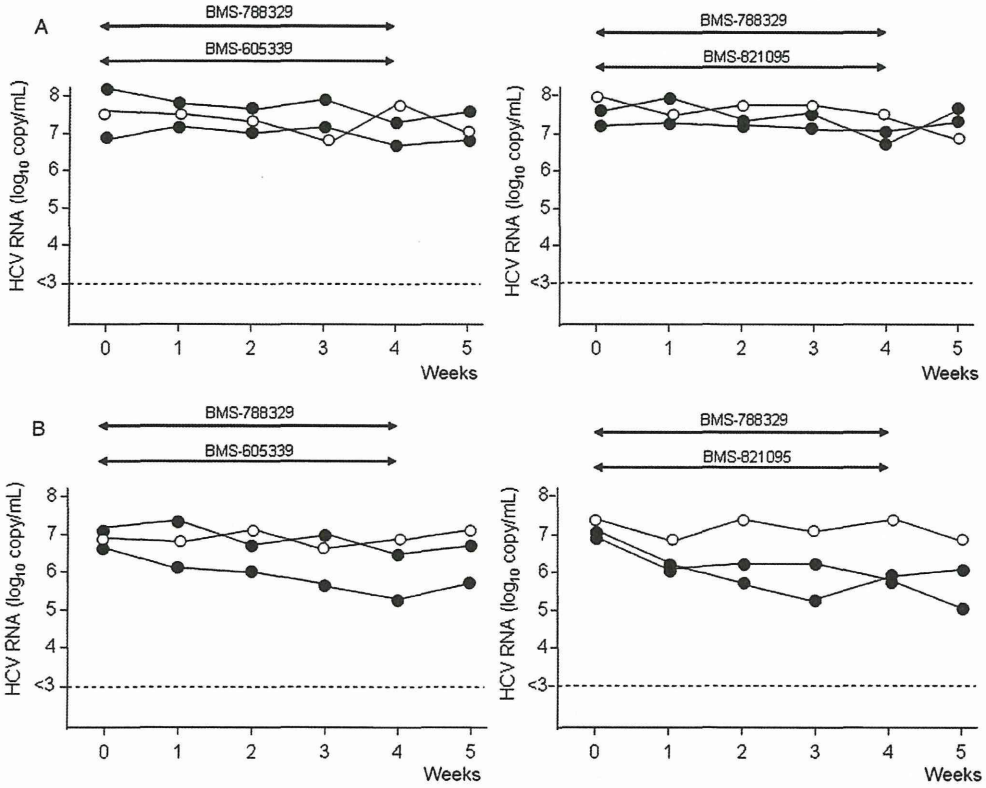

Figure 4 Antiviral effects of NS5A replication complex inhibitor combinations with either NS3 protease inhibitor or NS5B inhibitor in mice infected with hepatitis C virus (HCV) genotype 2. Each of the four HCV genotype 2a (A) or 2b (B) infected mice were treated with 10 mg/kg of BMS-788329 combined with 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).

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