

3) 超免疫不全マウスNOGをベースにした新規のヒト肝細胞移植TK-NOGマウスを用いて、HBVおよびHCV感染ヒト肝細胞移植TK-NOGマウスのウイルス感染率、抗ウイルス薬の薬効を評価した。

## C. 結果

1) Genotype 1b型C型肝炎患者血清を投与し感染させたマウスに200 mg/kgのtelaprevir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。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。

2) 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変異が出現し二重耐性型となりbreakthroughが生じた。さらにこのマウスに非核酸型ポリメラーゼ阻害剤を投与したところ、一旦は血中HCV RNAの低下を認めたがHCVは再燃し、この際、三重耐性変異が出現していた。

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併用療法はgenotype 1b型HCVに対して有用な治療法であった。一方、genotype 2型では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効果はやや弱い。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

### 1. 論文発表

- 1) 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, Tateno-Mukaidani C, Yoshizato K, Matsui H, Kanai A, Inaba T, McPhee F, Gao M, Chayama K. Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice. *Gut*. 2013 Jul;62:1055-61
- 2) Abe H, Hayes CN, Hiraga N, Imamura M, Tsuge M, Miki D, Takahashi S, Ochi H, Chayama K. A Translational Study of Resistance Emergence Using Sequential Direct-Acting Antiviral Agents for Hepatitis C Using Ultra-Deep Sequencing. *Am J Gastroenterol* 108:1464-72, 2013
- 3) Kosaka K, Hiraga N, Imamura M, Yoshimi S, Murakami E, Nakahara T, Honda Y, Ono A, Kawaoka T, Tsuge M, Abe H, Nelson Hayes C, Miki D, Aikata H, Ochi H, Ishida Y, Tateno C, Yoshizato K, Sasaki T, Chayama K. A novel TK-NOG based humanized mouse model for the study of HBV and HCV infections. *Biochem Biophys Res Commun*. 2013;441(1):230-5.
- 4) Sainz B Jr, Barretto N, Martin DN, Hiraga N, Imamura M, Hussain S, Marsh KA, Yu X, Chayama K, Alrefai WA, Uprichard SL. Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor. *Nat Med* 18(2); 281-5, 2012
- Hiraga N, Abe H, Imamura M, Tsuge M, Takahashi S, Hayes CN, Ochi H, Tateno C, Yoshizato K, Nakamura Y, Kamatani N, Chayama K. Impact of viral amino acid substitutions and host IL28B polymorphism on replication and susceptibility to interferon of hepatitis C virus. *Hepatology* 54(3); 764-71, 2011
- 5) Hiraga N, Imamura M, Abe H, Nelson Hayes C, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, Yoshizato K, Matsui H, Kanai A, Inaba T, Tanaka S, Chayama K. Rapid emergence of telaprevir resistant hepatitis C virus strain from wild type clone in vivo. *Hepatology* 54:781-8, 2011
- 6) Tsuge M, Fujimoto Y, Hiraga N, Zhang Y, Ohnishi M, Kohno T, Abe H, Miki D, Imamura M, Takahashi S, Ochi H, Hayes CN, Miya F, Tsunoda T, Chayama K. Hepatitis C virus infection suppresses the interferon response in the liver of the human hepatocyte chimeric mouse. *PLoS One*. 2011;6(8):e23856.
- 7) Ohara E, Hiraga N, Imamura M, Iwao E, Kamiya N, Yamada I, Kono T, Onishi M, Hirata D, Mitsui F, Kawaoka T, Tsuge M, Takahashi S, Abe H, Hayes CN, Ochi H, Tateno C, Yoshizato K, Tanaka S, Chayama K. Elimination of Hepatitis C Virus by Short Term NS3-4A and NS5B Inhibitor Combination Therapy in Human Hepatocyte Chimeric Mice. *J Hepatol*. 2011;54(5):872-8.
- 8) Abe H, Imamura M, Hiraga N, Tsuge M, Mitsui F, Kawaoka T, Takahashi S, Ochi H, Maekawa T, Hayes CN, Tateno C, Yoshizato K, Murakami S, Yamashita N, Matsuhira T, Asai K, Chayama K. ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both in vitro and in vivo. *J Hepatol*. 2011;55:11-8.

### 2. 学会発表

- 1) Michio Imamura, Hiromi Abe, C. Nelson Hayes, Nobuhiko Hiraga, Tomokazu Kawaoka, Masataka

Tsuge, Yoshiiku Kawakami, Hiroshi Aikata, Shoichi Takahashi, Kazuaki Chayama. Deep sequencing analysis of hepatitis C virus quasipieces in patients treated with telaprevir in combination with peginterferon and ribavirin. The 10th JSH Single Topic Conference, Tokyo. November 21, 2012

2) 今村 道雄, 阿部 弘美, 平賀 伸彦, 越智 秀典, 茶山 一彰. C型肝炎ウイルスの感染およびIFN治療におけるIL28B遺伝子多型の影響. 第77回インターフェロン・サイトカイン学術集会 2012年6月21日, 神戸

3) Hiraga N, Imamura M, Abe H, Hayes CN, Tsuge M, Takahashi T, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, Yoshizato K, Matsui H, Kanai A,

Inaba T, Chayama K. Rapid Emergence of Telaprevir Resistant Hepatitis C Virus Strain From Wild Type Clone in Vivo. 12th AASLD, San Francisco. November 4, 2011

4) Imamura M, Abe H, Hiraga N, Tsuge M, Takahashi S, C. Hayes CN, Ochi H, Tateno C, Yoshizato K, Chayama K. Impact of Viral Amino Acid Substitutions and Host IL28B polymorphism on Replication and Susceptibility to Interferon of Hepatitis C Virus. The 62th Annual Meeting of the American Association for the Study of Liver Diseases. San Francisco, 2011

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  
(平成23～25年度)

## 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, <u>Imamura M</u> , Hayes CN, Zhang Y, Kosaka K, Okazaki A, Murakami E, Tsuge M, Abe H, Aikata H, Takahashi S, Ochi H, Tateno-Mukaidani C, Yoshizato K, Matsui H, Kanai A, Inaba T, McPhee F, Gao M, Chayama K	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
Abe H, Hayes CN, Hiraga N, <u>Imamura M</u> , Tsuge M, Miki D, Takahashi S, Ochi H, Chayama K.	A Translational Study of Resistance Emergence Using Sequential Direct-Acting Antiviral Agents for Hepatitis C Using Ultra-Deep Sequencing	Am J Gastroenterol	108	1464-72	2013
Kosaka K, Hiraga N, <u>Imamura M</u> , Yoshimi S, Murakami E, Nakahara T, Honda Y, Ono A, Kawaoka T, Tsuge M, Abe H, Nelson Hayes C, Miki D, Aikata H, Ochi H, Ishida Y, Tateno C, Yoshizato K, Sasaki T, Chayama K	A novel TK-NOG based humanized mouse model for the study of HBV and HCV infections	Biochem Biophys Res Commun	441	230-5	2013
Hayes CN, Imamura M, Aikata H, <u>Chayama K</u>	Genetics of IL28B and HCV-response to infection and treatment.	Nat Rev Gastroenterol Hepatol	9(7)	406-17	2012
<u>Chayama K</u> , Hayes CN, <u>Imamura M</u> .	Impact of interleukin-28B genotype on in vitro and in vivo systems of hepatitis C virus replication	Hepatol Res	42(9)	841-53	2012
Miki D, Ohishi W, Ochi H, Hayes CN, Abe H, Tsuge M, Imamura M, Kamatani N, Nakamura Y, <u>Chayama K</u> .	Serum PAI-1 is a novel predictor for response to pegylated interferon- $\alpha$ -2b plus ribavirin therapy in chronic hepatitis C virus infection.	J Viral Hepat	19(2)	e126-e133	2012
Sainz B Jr, Barretto N, Martin DN, Hiraga N, Imamura M, Hussain S, Marsh KA, Yu X, Chayama K, Alrefai WA, Uprichard SL.	Identification of the Niemann-Pick C1-like 1 cholesterol absorption receptor as a new hepatitis C virus entry factor	Nat Med	18(2)	281-5	2012
Hiraga N, Abe H, <u>Imamura M</u> , Tsuge M, Takahashi S, Hayes CN, Ochi H, Tateno C, <u>Yoshizato K</u> , Nakamura Y, Kamatani N, <u>Chayama K</u>	Impact of viral amino acid substitutions and host IL28B polymorphism on replication and susceptibility to interferon of hepatitis C virus.	Hepatology	54(3)	764-71	2011

Hiraga N, <u>Imamura M</u> , Abe H, Nelson Hayes C, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, <u>Yoshizato K</u> , Matsui H, Kanai A, Inaba T, Tanaka S, <u>Chayama K</u> .	Rapid emergence of telaprevir resistant hepatitis C virus strain from wild type clone in vivo.	Hepatology	54(3)	781-88	2011
<u>Chayama K</u> , Hayes CN, Hiraga N, Abe H, Tsuge M, <u>Imamura M</u> .	Animal model for study of human hepatitis viruses.	J Gastroenterol Hepatol.	26	13-18s	2011
Tsuge M, Fujimoto Y, Hiraga N, Zhang Y, Ohnishi M, Kohno T, Abe H, Miki D, <u>Imamura M</u> , Takahashi S, Ochi H, Hayes CN, Miya F, Tsunoda T, <u>Chayama K</u>	Hepatitis C virus response in the liver of the human hepatocyte chimeric mouse.	PLoS One.	6	e23856.	2011
Tsuge M, Takahashi S, Hiraga N, Fujimoto Y, Zhang Y, Mitsui F, Abe H, Kawaoka T, <u>Imamura M</u> , Ochi H, Hayes CN, <u>Chayama K</u> .	Effects of hepatitis B virus infection on the interferon response in immunodeficient human hepatocyte chimeric mice.	J Infect Dis	2	224-8	2011
Saeed M, Shiina M, Date T, Akazawa D, Watanabe N, Murayama A, Suzuki T, Watanabe H, Hiraga N, <u>Imamura M</u> , <u>Chayama K</u> , Choi Y, Krawczynski K, Liang TJ, <u>Wakita T</u> , Kato T.	In Vivo adaptation of hepatitis C virus for efficient virus production and evasion of apoptosis.	Hepatology	54(2)	425-33	2011
Ohara E, Hiraga N, <u>Imamura M</u> , Iwao E, Kamiya N, Yamada I, Kono T, Onishi M, Hirata D, Mitsui F, Kawaoka T, Tsuge M, Takahashi S, Abe H, Hayes CN, Ochi H, Tateno C, <u>Yoshizato K</u> , Tanaka S, <u>Chayama K</u>	Elimination of Hepatitis C Virus by Short Term NS3-4A and NS5B Inhibitor Combination Therapy in Human Hepatocyte Chimeric Mice.	J Hepatol	54(5)	872-8	2011
Abe H, <u>Imamura M</u> , Hiraga N, Tsuge M, Mitsui F, Kawaoka T, Takahashi S, Ochi H, Maekawa T, Hayes CN, Tateno C, <u>Yoshizato K</u> , Murakami S, Yamashita N, Matsuhira T, Asai K, <u>Chayama K</u>	ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both in vitro and in vivo.	J Hepatol	55(1)	11-8	2011
Hata T, Uemoto S, Fujimoto Y, Murakami T, Tateno C, <u>Yoshizato K</u> , Kobayashi E.	Transplantation of engineered chimeric liver with autologous hepatocytes and xenobiotic scaffold.	Ann Surg	257(3)	542-7	2013
Tanaka F, Tominaga K, Sasaki E, Sogawa M, Yamagami H, Tanigawa T, Shiba M, Watanabe K, Watanabe T, Fujiwara Y, Kawada N, <u>Yoshizato K</u> , Arakawa T.	Cytoglobin May Be Involved in the Healing Process of Gastric Mucosal Injuries in the Late Phase Without Angiogenesis.	Dig Dis Sci	Jan 10.	Epub ahead of print	2013

Kosaka K, Hiraga N, Imamura M, Yoshimi S, Murakami E, Nakahara T, Honda Y, Ono A, Kawaoka T, Tsuge M, Abe H, Hayes CN, Miki D, Aikata H, Ochi H, Ishida Y, Tateno C, <u>Yoshizato K</u> , Sasaki T, Chayama K.	A novel TK-NOG based humanized mouse model for the study of HBV and HCV infections.	Biochem Biophys Res Commun	Nov;8441(1)	230-5	2013
<u>Yoshizato K</u> , Tateno C.	A mouse with humanized liver as an animal model for predicting drug effects and for studying hepatic viral infection: where to next?	Expert Opin Drug Metab Toxicol	Nov;9(11)	1419-35	2013
Tachibana A, Tateno C, <u>Yoshizato K</u> .	Repopulation of the immunosuppressed retrorsine-treated infant rat liver with human hepatocytes.	Xenotransplantation	Jul-Aug;20(4)	227-38	2013
Shi N, Hiraga N, Imamura M, Hayes CN, Zhang Y, Kosaka K, Okazaki, Murakami E, Tsuge M, Abe H, Aikata H, Takahashi S, Ochi H Tateno-Mukaidani C <u>Yoshizato K</u> , Matsui H, Kanai A, Inaba T, McPhee F, Gao M, Chayama K.	Combination therapies with NS5A, NS3 and NS5B inhibitors on different genotypes of hepatitis C virus in human hepatocyte chimeric mice.	Gut	Jul62(7)	1055-61	2013
Tateno C, Miya F, Wake K, Kataoka M, Ishida Y, Yamasaki C, Yanagi A, Kakuni M, Wisse E, Verheyen F, Inoue K, Sato K, Kudo A, Arii S, Itamoto T, Asahara T, Tsunoda T, <u>Yoshizato K</u> .	Morphological and microarray analyses of human hepatocytes from xenogeneic host livers.	Lab Invest	Jan93(1)	54-71	2013
Fujiwara S, Fujioka H, Tateno C, Taniguchi K, Ito M, Ohishi H, Utoh R, Ishibashi H, Kanematsu T, <u>Yoshizato K</u> .	A novel animal model for in vivo study of liver cancer metastasis.	World J Gastroenterol	Aug718(29)	3875-82	2012
Izuka M, Ogawa T, Enomoto M, Motoyama H, <u>Yoshizato K</u> , Ikeda K, Kawada N.	Induction of microRNA-214-5p in human and rodent liver fibrosis.	Fibrogenesis Tissue Repair	Aug15(1)	12	2012
Tatsumi K, Ohashi K, Tateno C, <u>Yoshizato K</u> , Yoshioka A, Shima M, Okano T.	Human hepatocyte propagation system in the mouse livers: functional maintenance of the production of coagulation and anticoagulation factors.	Cell Transplant	21(2-3)	437-45	2012

Ohashi K, Tatsumi K, Tateno C, Kataoka M, Utoh R, Yoshizato K, Okano T.	Liver tissue engineering utilizing hepatocytes propagated in mouse livers in vivo.	Cell Transplant	21(2-3)	429-36	2012
Okazaki A, Hiraga N, Imamura M, Hayes CN, Tsuge M, Takahashi S, Aikata H, Abe H, Miki D, Ochi H, Tateno C, <u>Yoshizato K</u> , Ohdan H, Chayama K.	Severe necroinflammatory reaction caused by natural killer cell-mediated Fas/Fas ligand interaction and dendritic cells in human hepatocyte chimeric mouse.	Hepatology	Aug 56(2)	555-66	2012
Ogawa T, Enomoto M, Fujii H, Sekiya Y, <u>Yoshizato K</u> , Ikeda K, Kawada N.	MicroRNA-221/222 upregulation indicates the activation of stellate cells and the progression of liver fibrosis.	Gut	Nov 61(11)	1600-9	2012
<u>Yoshizato K</u> , Tateno C, Utoh R.	Mice with liver composed of human hepatocytes as an animal model for drug testing.	Curr Drug Discov Technol	Mar 9(1)	63-76	2012
Sekiya Y, Ogawa T, <u>Yoshizato K</u> , Ikeda K, Kawada N.	Suppression of hepatic stellate cell activation by microRNA-29b.	Biochem Biophys Res Commun	Aug 19 412(1)	74-9	2011
Sekiya Y, Ogawa T, Iizuka M, <u>Yoshizato K</u> , Ikeda K, Kawada N.	Down-regulation of cyclin E1 expression by microRNA-195 accounts for interferon- $\beta$ -induced inhibition of hepatic stellate cell proliferation.	J Cell Physiol	Oct 226(10)	2535-42	2011
Thuy le TT, Morita T, Yoshida K, Wakasa K, Iizuka M, Ogawa T, Mori M, Sekiya Y, Momen S, Motoyama H, Ikeda K, <u>Yoshizato K</u> , Kawada N.	Promotion of liver and lung tumorigenesis in DEN-treated cytoglobin-deficient mice.	Am J Pathol	Aug 179(2)	1050-60	2011
Hiraga N, Imamura M, Abe H, Hayes CN, Kono T, Onishi M, Tsuge M, Takahashi S, Ochi H, Iwao E, Kamiya N, Yamada I, Tateno C, <u>Yoshizato K</u> , Matsui H, Kanai A, Inaba T, Tanaka S, Chayama K.	Rapid emergence of telaprevir resistant hepatitis C virus strain from wildtype clone in vivo.	Hepatology	Sep 2 54(3)	781-8	2011
Hiraga N, Abe H, Imamura M, Tsuge M, Takahashi S, Hayes CN, Ochi H, Tateno C, <u>Yoshizato K</u> , Nakamura Y, Kamatani N, Chayama K.	Impact of viral amino acid substitutions and host interleukin-28b polymorphism on replication and susceptibility to interferon of hepatitis C virus.	Hepatology	Sep 2 54(3)	764-71	2011
Amano H, Hino H, Tateno C, Emoto K, Imaoka Y, Yamasaki C, Itamoto T, Tashiro H, Asahara T, Ohdan H, <u>Yoshizato K</u> .	Therapeutic potential of propagated hepatocyte transplantation in liver failure.	J Surg Res	May 167(1)	e29-37	2011



Kuroki M., Ariumi Y., <u>Hijikata M.</u> , Ikeda M., Dansako H., Wakita T., Shimotohno K., Kato N.	PML tumor suppressor protein is required for HCV production	Biochem. Biophys. Res. Commun.	430	592-597	2013
Aly H.H., Shimotohno K., <u>Hijikata M.</u> , Seya T.	In vitro models for the analysis of HCV life cycle.	Microbiol. and Immunol.	56(1)	1-9	2012
Wakita T., Suzuki T., Evans M.J., Shimotohno K., Chayama K., Matsuura Y., <u>Hijikata M.</u> , Moriishi K., Seya T., Enomoto N., Koike K., Kato N., Kanto T., Hotta H.	Will there be an HCV meeting in 2020? Summary of the 17 <sup>th</sup> International Meeting in Hepatitis C Virus and Related Viruses	Gastroenterology	141(1)	E1-E5	2011
Ariumi, Y. Misao Kuroki M., Kushima Y., Osugi K., <u>Hijikata M.</u> , Maki M., Ikeda M., Kato N.	Hepatitis C virus hijacks P-body and stress granule components around lipid droplets.	J. Virol.,	85(14)	6882-6892	2011
Takahashi Y, Ando M, Nishikawa M, Hiraga N, Imamura M, Chayama K, <u>Takakura Y.</u>	Long-Term Elimination of Hepatitis C Virus from Human Hepatocyte Chimeric Mice After Interferon- $\gamma$ Gene Transfer.	Hum Gene Ther Clin Dev.	In press		2013
Uno S, Nishikawa M, Mohri K, Umeki Y, Matsuzaki N, Takahashi Y, Fujita H, Kadowaki N, <u>Takakura Y.</u>	Efficient delivery of immunostimulatory DNA to mouse and human immune cells through the construction of polypod-like structured DNA.	Nanomedicine	In press		2013
Mukumoto H, Takahashi Y, Ando M, Nishikawa M, <u>Takakura Y.</u>	Expression profile-dependent improvement of insulin sensitivity by gene delivery of interleukin-6 in a mouse model of type II diabetes.	Mol Pharm.	10(10)	3812-3821	2013
Miyakawa N, Nishikawa M, Takahashi Y, Ando M, Misaka M, Watanabe Y, <u>Takakura Y.</u>	Gene delivery of albumin binding peptide-interferon $\gamma$ fusion protein with improved pharmacokinetic properties and sustained biological activity.	J Pharm Sci.	102(9)	3110-3118	2013
Watcharanurak K, Nishikawa M, Takahashi Y, <u>Takakura Y.</u>	Controlling the kinetics of interferon transgene expression for improved gene therapy	J Drug Target	20(9)	764-769	2012
Watcharanurak K, Nishikawa M, Takahashi Y, Kabashima K, Takahashi R, <u>Takakura Y.</u>	Regulation of immunological balance by sustained interferon- $\gamma$ gene transfer for acute phase of atopic dermatitis in mice.	Gene Ther	20(5)	538-544	2013
Ando M, Takahashi Y, Nishikawa M, Watanabe Y, <u>Takakura Y.</u>	Constant and steady transgene expression of interferon- $\gamma$ by optimization of plasmid construct for safe and effective interferon- $\gamma$ gene therapy.	J Gene Med	14(4)	288-295	2012

#### IV. 研究成果の刊行物・別刷

(平成23～25年度)

## 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.

**INTRODUCTION**

Hepatitis C virus (HCV) infection is a major cause of chronic liver diseases, such as cirrhosis and hepatocellular carcinoma.<sup>1 2</sup> 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

**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.

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.<sup>3 4</sup> 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.<sup>5</sup> Following the INFORM-1 study, we and other groups have also reported that a DAA-only

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combination comprising NS3 protease inhibitor (PI), Bristol-Myers Squibb (BMS)-650032 (asunaprevir) and NSSA replication complex inhibitor (RCI), BMS-790052 (daclatasvir) can achieve high sustained virological response (SVR) rates in patients with HCV genotype 1b infection.<sup>6</sup> A number of DAA-only combination studies are now entering phase 2 clinical trials.<sup>7</sup> 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<sub>10</sub> 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.<sup>8</sup> 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.<sup>9</sup> This animal model is useful for evaluating anti-HCV drugs such as Peg-alfa and NS3 PI.<sup>10–11</sup> 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.<sup>12</sup> 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.<sup>13</sup>

### Determination of IC<sub>50</sub> in culture systems

The genotype 1b (Con 1) replicon cell line was constructed as described previously.<sup>14</sup> 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.<sup>15</sup> 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<sub>50</sub>), were determined as described previously.<sup>16</sup>

### 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<sup>+/+</sup>/SCID<sup>+/+</sup> mice and transplantation of human hepatocytes were performed as described previously.<sup>17</sup> 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<sub>10</sub> 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.<sup>11–12</sup> 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<sub>10</sub> 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.<sup>12</sup> The primers used to amplify the NS3 region were 5'-GTGCTCCAAGCTGGCATAAC-3' and 5'-AGGACCGAGGAATCGAACAT-3' as the first (outer) primer pair and 5'-CTAGAGTGCCGACTTTCGTG-3' and 5'-ACTGATCCCTGGAGGCGTAGC-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'-GATGGTAGTGCATGTCC CC-3' as the second (inner) primer pair. The primers used to amplify the NS5B region were 5'-TAAGCGAGGAGGCT GGTGAG-3' and 5'-CCTATTGGCCTGGAGTGTTC-3' as the first (outer) primer pair and 5'-GACTCAACGGTCC TGAGAG-3' and 5'-CCTATTGGCCTGGAGTGTTC-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)<sup>18</sup>.

### 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 <sub>50</sub> (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.<sup>19</sup> Direct sequencing consensus data were used to improve alignment to the reference sequence. Codon counts were merged and analysed using R V2.14.

#### 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

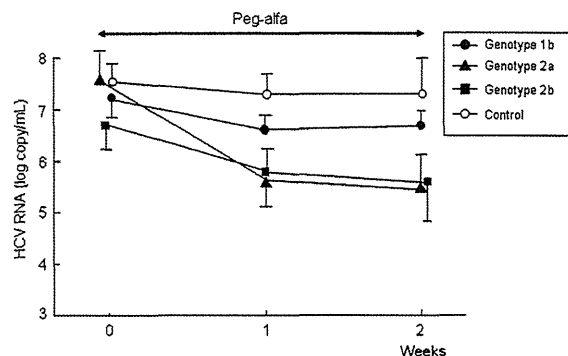
The inhibitory effects of BMS-605339, BMS-788329 and BMS-821095 on HCV replication were analysed *in vitro* using HCV replicon cells (genotype 1b, Con 1 and genotype 2a, JFH1). A summary of the IC<sub>50</sub> values for each drug is shown in table 1. Antiviral activities of BMS-605339 and BMS-788329 were similar to asunaprevir<sup>15</sup> and daclatasvir,<sup>20</sup> respectively. BMS-605339 and BMS-821095 IC<sub>50</sub> 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<sup>5</sup> 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<sub>10</sub> 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<sub>10</sub> and 1.5 log<sub>10</sub> 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<sup>5</sup> 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.

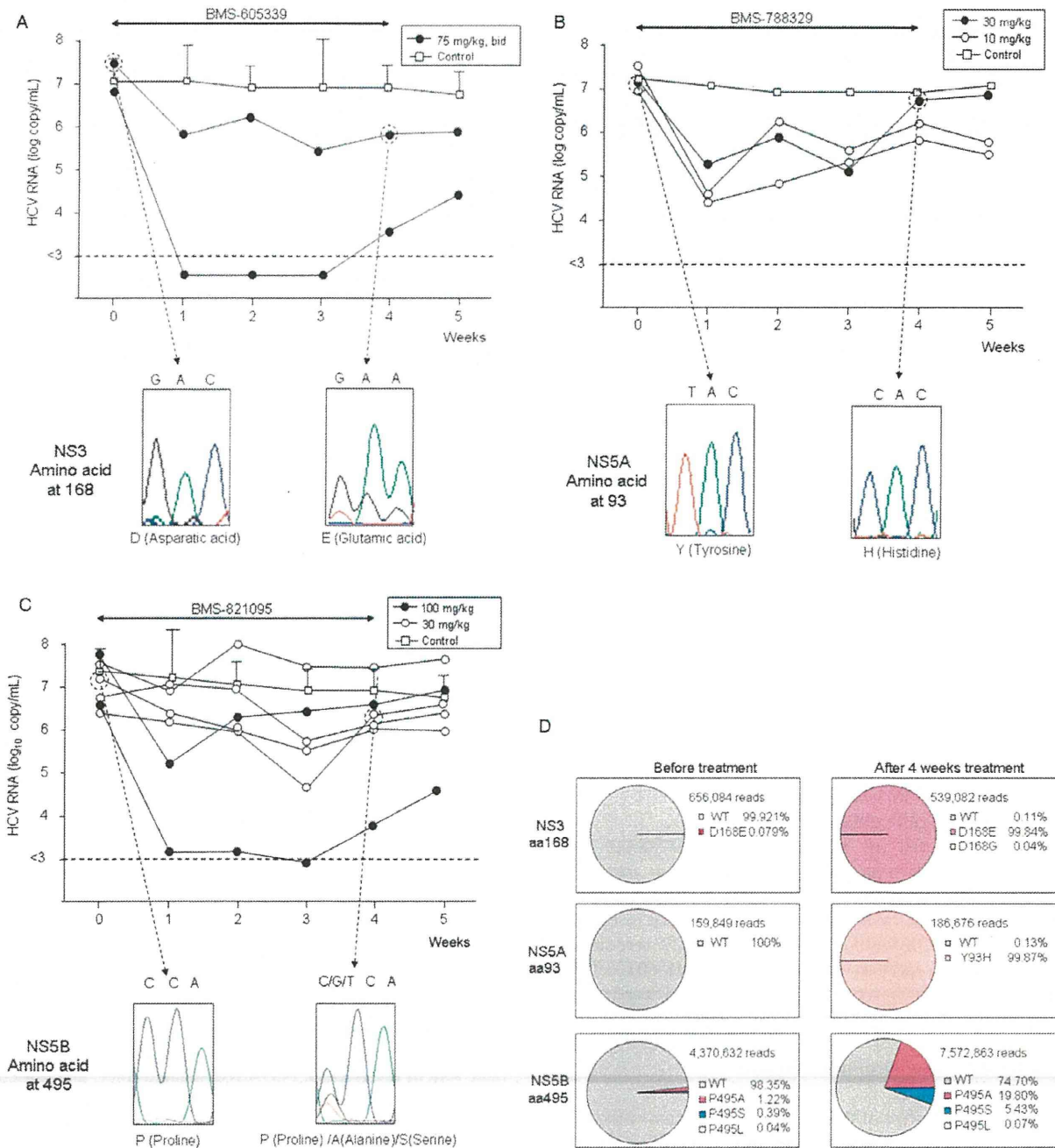
of HCV obtained from a patient infected with genotype 1b. Eight weeks after injection when stable viraemia had developed, mice were treated with BMS-605339 (NS3PI) (figure 2A), BMS-788329 (NS5A RCI) (figure 2B) or BMS-821095 (NS5B site I inhibitor) (figure 2C) for 4 weeks. Although all BMS-605339 and 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)<sup>21</sup> in a BMS-605339-treated mouse (figure 2A), and a mutation coding for Y93H in the NS5A region (NS5A RCI-resistant variant)<sup>14</sup> 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)<sup>22</sup> 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



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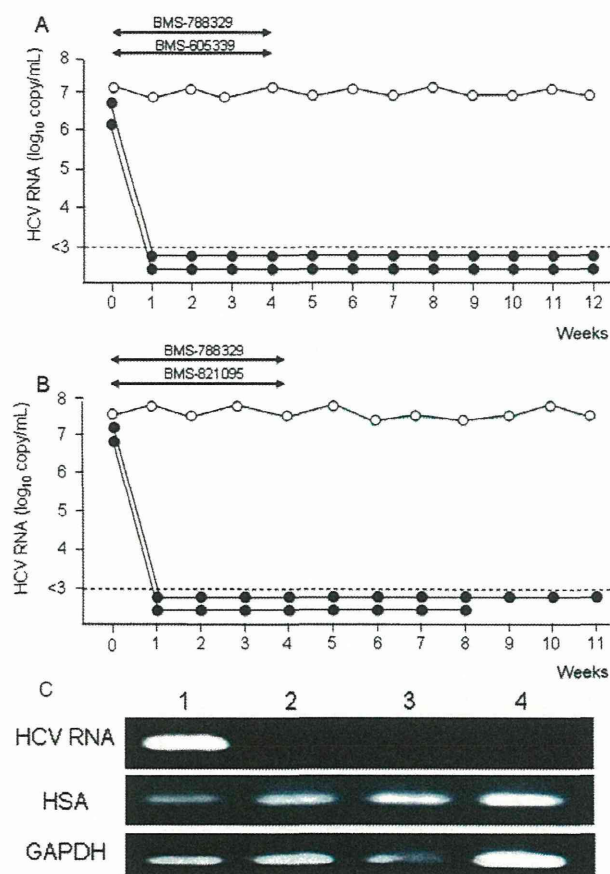
**Figure 2** Antiviral effects of BMS-605339, BMS-788329 or BMS-821095 monotherapy in mice infected with hepatitis C virus (HCV) genotype 1b. Mice were injected intravenously with  $10^5$  copies of HCV genotype 1b. Eight weeks after HCV infection, mice were treated with the indicated concentrations 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<sup>21</sup> and has already been observed in combination therapies with two DAA.<sup>5 23 24</sup> 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,<sup>12</sup> 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<sub>50</sub> 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.<sup>25–28</sup> 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.<sup>29</sup> 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.<sup>30–32</sup> Although BMS-788329 was very potent against the genotype-2a JFH-1 replicon (IC<sub>50</sub> 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<sub>50</sub> 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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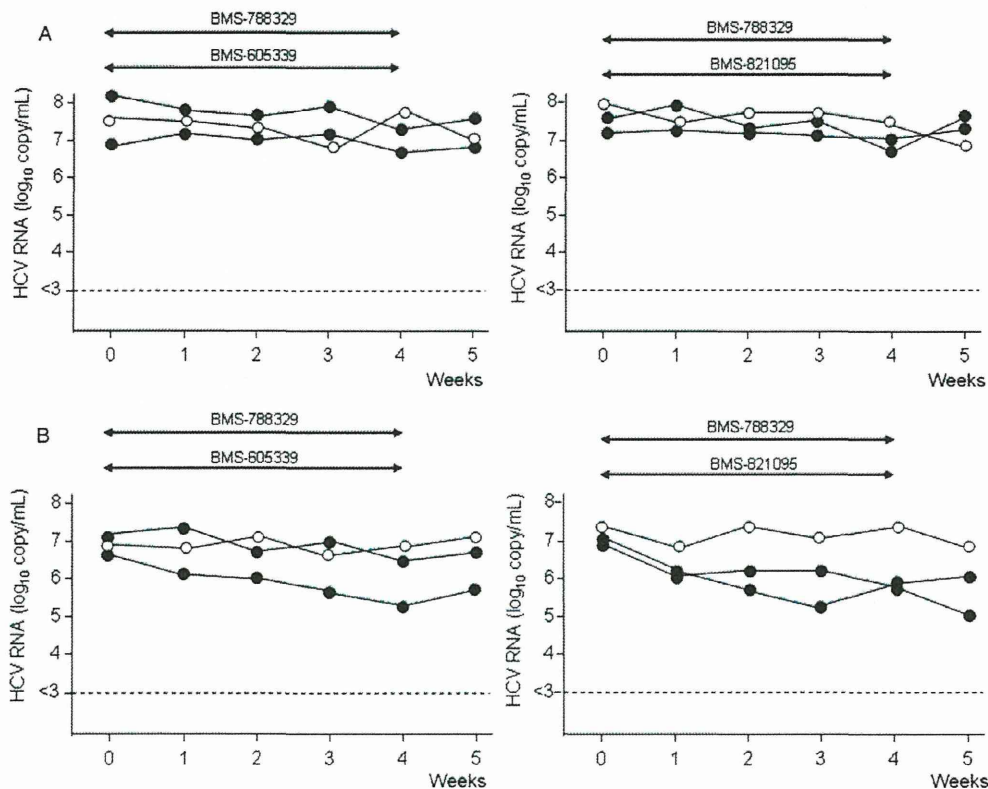
**Competing interests** MG and FM are employees of Bristol-Myers Squibb. All other authors declare no competing interests.

**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.



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**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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## REFERENCES

- 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.
- 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.
- 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.
- 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.
- 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.
- 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.
- Gane E. Future hepatitis C virus treatment: interferon-sparing combinations. *Liver Int* 2011;31(Suppl. 1):62–7.
- 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.
- Mercer DF, Schiller DE, Elliott JF, *et al.* Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001;7:927–33.
- 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.
- 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.
- 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.
- Don RH, Cox PT, Wainwright BJ, *et al.* 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res* 1991;19:4008.
- 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.
- 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.
- 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.
- 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.
- 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.
- Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics* 2010;26:589–95.
- 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.
- Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* 2010;138:447–62.
- Kukulj 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.
- 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.
- 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.



- 25 Paulson MS, Yang H, Shih IH, *et al.* Comparison of HCV NS3 protease and NS5B polymerase inhibitor activity in 1a, 1b and 2a replicons and 2a infectious virus. *Antiviral Res* 2009;83:135–42.
- 26 Imhof I, Simmonds P. Genotype differences in susceptibility and resistance development of hepatitis C virus to protease inhibitors telaprevir (VX-950) and danoprevir (ITMN-191). *Hepatology* 2011;53:1090–9.
- 27 Imhof I, Simmonds P. Development of an intergenotypic hepatitis C virus (HCV) cell culture method to assess antiviral susceptibilities and resistance development of HCV NS3 protease genes from HCV genotypes 1 to 6. *J Virol* 2010;84:4597–610.
- 28 Scheel TK, Gottwein JM, Mikkelsen LS, *et al.* Recombinant HCV variants with NS5A from genotypes 1–7 have different sensitivities to an NS5A inhibitor but not interferon-alpha. *Gastroenterology* 2011;140:1032–42.
- 29 Reiser M, Hinrichsen H, Benhamou Y, *et al.* Antiviral efficacy of NS3-serine protease inhibitor BILN-2061 in patients with chronic genotype 2 and 3 hepatitis C. *Hepatology* 2005;41:832–5.
- 30 Lenz O, Verbinen T, Lin TI, *et al.* In vitro resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. *Antimicrob Agents Chemother* 2010;54:1878–87.
- 31 Cheng G, Chan K, Yang H, *et al.* Selection of clinically relevant protease inhibitor-resistant viruses using the genotype 2a hepatitis C virus infection system. *Antimicrob Agents Chemother* 2011;55:2197–205.
- 32 Delang L, Vliegen I, Froeyen M, *et al.* Comparative study of the genetic barriers and pathways towards resistance of selective inhibitors of hepatitis C virus replication. *Antimicrob Agents Chemother* 2011;55:4103–13.



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

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

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- OBJECTIVES:** Direct-acting antiviral agents (DAAs) against hepatitis C virus (HCV) have recently been developed and are ultimately hoped to replace interferon-based therapy. However, DAA monotherapy results in rapid emergence of resistant strains and DAAs must be used in combinations that present a high genetic barrier to resistance, although viral kinetics of multidrug-resistant strains remain poorly characterized. The aim of this study is to track the emergence and fitness of resistance using combinations of telaprevir and NS5A or NS5B inhibitors with genotype 1b clones.
- METHODS:** HCV-infected chimeric mice were treated with DAAs, and resistance was monitored using direct and ultra-deep sequencing.
- RESULTS:** Combination therapy with telaprevir and BMS-788329 (NS5A inhibitor) reduced serum HCV RNA to undetectable levels. The presence of an NS3-V36A telaprevir resistance mutation resulted in poor response to telaprevir monotherapy but showed significant HCV reduction when telaprevir was combined with BMS-788329. However, a BMS-788329-resistant strain emerged at low frequency. Infection with a BMS-788329-resistant NS5A-L31V mutation rapidly resulted in gain of an additional NS5A-Y93A mutation that conferred telaprevir resistance during combination therapy. Infection with dual NS5A-L31V/NS5A-Y93H mutations resulted in poor response to combination therapy and development of telaprevir resistance. Although HCV RNA became undetectable soon after the beginning of combination therapy with BMS-788329 and BMS-821095 (NS5B inhibitor), rebound with emergence of resistance against all three drugs occurred. Triple resistance also occurred following infection with the NS3V36A/NS5AL31V/NS5AY93H triple mutation.
- CONCLUSIONS:** Resistant strains easily develop from cloned virus strains. Sequential use of DAAs should be avoided to prevent emergence of multidrug-resistant strains.

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

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## INTRODUCTION

According to the 2012 World Health Organization report, approximately 150 million individuals are chronically infected with hepatitis C virus (HCV) worldwide (1). Chronic HCV infection leads to chronic hepatitis, liver cirrhosis, liver failure, and hepatocellular carcinoma (1). Recently, two direct-acting antiviral agents (DAAs), telaprevir and boceprevir, have been approved for use in daily clinical practice to treat patients chronically infected

with HCV genotype 1 (2–9). Triple therapy with peg-interferon, ribavirin, and either telaprevir or boceprevir has been reported to be the most effective approved treatment so far, with an eradication rate of 50 to 70%, compared with no >50% for combination therapy with peg-interferon and ribavirin alone (2–9). However, triple therapy is approved only for genotype 1, and many treated patients experience severe side effects that often result in early termination of the therapy (2–9). In an effort to establish safer

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

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

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

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

## METHODS

### Animals

Generation of the uPA<sup>+/+</sup>/SCID<sup>+/+</sup> mice and transplantation of human hepatocytes were performed as described

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

### Reagents

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

### Human serum samples

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

### Construction of mutant HCV strains and HCV RNA synthesis

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

### RNA extraction and HCV RNA quantification

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