

Doctor

José Luis Salazar

Director de División de Nuevas Creaciones (C)

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO

E. _____ S. _____

D.

SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



No. 12-059496- -00004-0000

Fecha: 2014-04-14 16:33:58 Dep. 2020 DIR.NUEVASCR
Tra. 11 PATENTEIYII Eve: 378 FASENACIONALI
Act. 523 RESPUESTA Folios: 22

Referencia: Expediente No. 12.059.496

Solicitud de Patente de Invención titulada: "Combinaciones de un inhibidor específico de NS5A del HCV e inhibidor de proteasa NS3 del HCV"

Solicitante: Bristol-Myers Squibb Company

RESPUESTA AL AUTO NOTIFICADO POR FIJACIÓN EN LISTA

DEL 21 DE ENERO DE 2014, OFICIO NO. 236 (EXAMEN DE FONDO)

ÁLVARO CORREA ORDÓÑEZ, mayor de edad y vecino de Bogotá D.C., identificado tal como aparece al pie de mi firma, en mi carácter de apoderado de la sociedad en referencia, doy respuesta al auto emanado por ese Despacho dentro del término legal:

1. Objeciones del Examinador

1.1 Descripción (Art 28 D 486)

El examinador establece que la descripción no divulga el efecto sinérgico de la combinación de un inhibidor de NS3/4A de fórmula (II), un inhibidor NS5A de fórmula (I) y ribavirina de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla.

1.2 Nivel inventivo

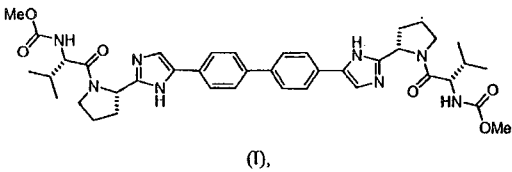
El examinador establece que los siguientes documentos D1 y D2 afectan el nivel inventivo de las reivindicaciones 1 a 9 de la solicitud en estudio:

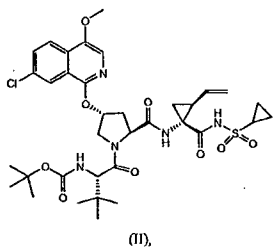
Ítem	Documento	Título	Fecha de Publicación
D1	WO2009085659	"Crystalline forms of n-(tert-butoxycarbonyl)-3-methyl-L-valyl-(4R)-4-((7-chloro-4-methoxy-1-isoquinolinyloxy)-n-((1R,2S)-1-((cyclopropylsulfonyl)carbamoyl)-2-vinylcyclopropyl)-L-prolinamide"	09/Julio/2009

Ítem	Documento	Título	Fecha de Publicación
D2	WO2009020828	"Crystalline form of methyl ((1S)-1-(((2S)-2-(5-(4'-(2-((2S)-1-((2S)-2-(methoxycarbonyl)amino)-3-methylbutanoyl)-2-pyrrolidinyl)-1H-imidazol-5-yl)-4-biphenyl)-1H-imidazol-2-yl)-1-pyrrolidinyl)carbonyl)-2-methylpropyl)carbamate dihydrochloride salt"	12/Febrero/2009

Y argumenta en forma resumida lo siguiente:

La reivindicación 1 consiste en una composición que comprende una cantidad terapéuticamente efectiva de un inhibidor HCV específico de fórmula (I) o una sal del mismo y una cantidad terapéuticamente efectiva de un inhibidor de proteasa NS3 del HCV específico de fórmula (II) o una sal del mismo y un portador farmacéuticamente aceptable.





El examinador efectúa la siguiente comparación entre la reivindicación 1 y los documentos D1 y D2:

Características esenciales	D2	D1
Una composición que comprende:	Una composición que comprende:	Una composición que comprende:
i) una cantidad terapéuticamente efectiva de un inhibidor HCV específico de fórmula (I) o una sal del mismo	un inhibidor de NS5A para el tratamiento del HCV de fórmula (I) (Pág. 1, Líneas 12 a 18)	No menciona
una cantidad terapéuticamente efectiva de un inhibidor de proteasa NS3 del HCV específico de fórmula (II) o una sal del mismo	un inhibidor de serina proteasa NS3 de HCV, tal como telaprevir (Pág. 17, Tabla 1)	un inhibidor de NS3/4A para el tratamiento del HCV de fórmula (II) (Pág. 1, Líneas 10 a 16)
y un portador farmacéuticamente aceptable.	Un portador aceptable (Pág. 15, Líneas 15 a 19)	Un portador aceptable (Pág. 18, Líneas 5 a 9)

El documento D2 se considera el estado de la técnica cercano a la invención definida en la reivindicación 1 porque el documento D2 divulga combinaciones y composiciones del compuesto de fórmula (I) un inhibidor de NS5A útil en el tratamiento del HCV (Pág. 1, Líneas 12 a 18) y un inhibidor de serina proteasa NS3 de HCV, tal como telaprevir (Pág. 17, Tabla 1). D2 señala que las combinaciones del compuesto de fórmula (II) con el inhibidor de serina proteasa puede resultar en un efecto sinérgico en la actividad citotóxica frente al HCV por la combinación de los mecanismos de acción de los fármacos (Pág. 8, Líneas 15 a 24).

Por su parte, el documento D1 divulga el compuesto de fórmula (II) un inhibidor de NS3/4A para el tratamiento del HCV (Pág. 1, Líneas 10 a 16) y composiciones del mismo.

Las diferencias entre la invención, definida en la reivindicación 1 y la composición divulgada en el documento D2 consiste en que D2 no enseña específicamente el inhibidor NS3 de fórmula (II).

Las diferencias entre la invención, definida en la reivindicación 1 y la composición divulgada en el documento D1 consisten en que D1 no enseña la incorporación de un inhibidor NS5A.

El efecto que se logra por el hecho de combinar el inhibidor NS5A de fórmula (I) y el inhibidor NS3 de fórmula (II) es obtener un efecto sinérgico en la actividad citotóxica frente al HCV.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo modificar una combinación sinérgica de un inhibidor NS5A de fórmula (I) y un inhibidor NS3 de HCV divulgada en D2 empleando el inhibidor NS3 de HCV de fórmula (II) divulgado en D1 para obtener una composición con actividad citotóxica sinérgica frente al HCV.

En consecuencia, la persona del oficio normalmente versada contaba con la sugerencia o motivación razonable para modificar la combinación sinérgica de un inhibidor de NS5A de fórmula (I) y un inhibidor de serina proteasa NS3 de HCV, tal como telaprevir (Pág. 17, Tabla 1) divulgada en D2, cambiando el inhibidor de serina proteasa NS3 telaprevir por el inhibidor de serina proteasa NS3 de fórmula (II) divulgado en D1, por lo cual la materia reclamada se considera obvia.

En conclusión, la reivindicación 1 no cumple con el requisito de nivel inventivo (Art.18) y puesto que las reivindicaciones 2 a 6 no reivindican elementos esenciales que la hagan inventiva, dichas reivindicaciones tampoco cumplen con el citado requisito.

Reivindicación 7

Con relación a la reivindicación 7 el examinador efectúa la siguiente comparación entre dicha reivindicación y los documentos D1 y D2.

Características esenciales	D1	D2
Una composición que comprende:	Una composición que comprende:	Una composición que comprende:
ii) una cantidad terapéuticamente efectiva de un inhibidor HCV específico de fórmula (I) o una sal del mismo	No menciona	un inhibidor de NS5A para el tratamiento del HCV de fórmula (I) (Pág. 1, Líneas 12 a 18)

Características esenciales	D1	D2
una cantidad terapéuticamente efectiva de un inhibidor de proteasa NS3 del HCV específico de fórmula (II) o una sal del mismo	un inhibidor de NS3/4A para el tratamiento del HCV de fórmula (II) (Pág. 1, Líneas 10 a 16)	un inhibidor de serina proteasa NS3 de HCV, tal como telaprevir (Pág. 17, Tabla 1)
uno o dos compuestos adicionales con actividad anti-HCV donde al menos uno de los compuestos es un interferón o ribavirina	interferón α2b y ribavirina (Pág. 18, Tabla 1)	No menciona
y un portador farmacéuticamente aceptable.	Un portador aceptable (Pág. 18, Líneas 5 a 9)	Un portador aceptable (Pág. 15, Líneas 15 a 19)

El documento D1 se considera el estado de la técnica cercano a la invención definida en la reivindicación 7 porque el documento D1 divulga el compuesto de fórmula (II) un inhibidor de NS3/4A para el tratamiento del HCV (Pág. 1, Líneas 10 a 16) y composiciones del mismo. Específicamente D1 divulga la combinación con interferón α2b/ribavirina, entre otros (Pág. 18, Tabla 1).

Por su parte, el documento D2 divulga combinaciones y composiciones del compuesto de fórmula (I) un inhibidor de NS5A útil en el tratamiento del HCV (Pág.1, Líneas 12 a 18) y un inhibidor de serina proteasa NS3 de HCV, tal como telaprevir (Pág. 17, Tabla 1). D2 señala que las combinaciones del compuesto de fórmula (II) con el inhibidor de serina proteasa puede resultar en un efecto sinérgico en la actividad citotóxica frente al HCV por la combinación de los mecanismos de acción de los fármacos (Pág. 8, Líneas 15 a 24).

Las diferencias entre la invención, definida en la reivindicación 1 y la composición divulgada en el documento D1 consisten en que D1 no enseña la incorporación de un inhibidor NS5A.

Las diferencias entre la invención, definida en la reivindicación 1 y la composición divulgada en el documento D2 consiste en que D2 no enseña específicamente el inhibidor NS3 de fórmula (II) y la combinación concomitante con otros fármacos anti-HCV.

El efecto que se logra por el hecho de combinar el inhibidor NS5A de fórmula (I), un inhibidor NS3 de fórmula (II) y al menos un compuesto anti-HCV seleccionado de interferón o ribavirina es obtener una composición con actividad citotóxica sinérgica frente al HCV.

Por lo tanto, el problema técnico objetivo que pretende resolver esta invención se puede formular así: cómo modificar una composición sinérgica de un inhibidor de NS3/4A de fórmula (II), interferón y ribavirina divulgada en D1 incorporando el inhibidor NS5A de fórmula (I) divulgado en la composición de D2, para obtener una composición con actividad citotóxica sinérgica frente al HCV.

En consecuencia, la persona del oficio normalmente versada contaba con la sugerencia o motivación razonable para modificar la composición sinérgica de un inhibidor de NS3/4A de fórmula (II), interferón y ribavirina divulgada en D1, incorporando el inhibidor NS5A de fórmula (I) divulgado en D2, por lo cual la materia reclamada se considera obvia.

Concluye el examinador que las reivindicaciones 7,8 y 9 no cumplen con el requisito de nivel inventivo.

2. Argumentos a favor de la patentabilidad de la solicitud

2.1 Descripción

Con relación a la observación de su Despacho en cuanto a que la descripción no divulga el efecto sinérgico de la combinación de un inhibidor de NS3/4A de fórmula (II), un inhibidor

NS5A de fórmula (I) y ribavirina de manera suficientemente completa para que la persona del oficio normalmente versada en la materia pueda ejecutarla, mi representada manifiesta que esta en desacuerdo ya que en lo largo de la descripción se presentan ejemplos que sustentan la invención. Adicionalmente, con el presente escrito se anexan datos comparativos que claramente demuestran que la combinación de la solicitud en estudio es inesperada y sorprendente lo cual le otorga altura inventiva, tal como se demuestra a continuación.

2.2 Nivel inventivo

El nivel inventivo de una solicitud se encuentra definido en el Artículo 18 de la Decisión 486 y se establece cuando *“si para una persona del oficio normalmente versada en la materia técnica correspondiente, esa invención no hubiese resultado obvia ni se hubiese derivado de manera evidente del estado de la técnica”*.

Contrario a lo que plantea el Examinador, los documentos del arte previo citado no afectan el nivel inventivo de la presente solicitud por cuanto no es posible derivar la presente invención a partir de las enseñanzas presentadas en los documentos D1 y D2.

Al respecto, mi representada presenta los siguientes argumentos:

Es bien conocido en el campo del tratamiento de la Hepatitis C que los resultados de combinar diferentes tratamientos terapéuticos son impredecibles y que muchas combinaciones no proporcionan resultados sinérgicos. Aunque no se puede decir definitivamente que cada combinación fuera más que aditiva, los resultados muestran que estos fueron al menos aditivos y no demostraron antagonismo. Aunque no pueda esperarse para estas combinaciones que sean antagonistas, el principal problema con estas combinaciones es que no hay una expectativa sólida para ningún resultado dado.

El antagonismo es posible siempre que sean aditivas y sinérgicas. La naturaleza inesperada de combinar compuestos es lo que hace a la combinación exitosa inesperada e inventiva.

Desde que se presentó la solicitud se obtuvieron datos clínicos que muestran que casi la mitad de los pacientes a los que se les dio los dos componentes en la ausencia de interferona-alfa pegilada y ribavirina tenían una respuesta virológica completa a las 12

semanas, lo que significa que ellos no tenían HCV RNA detectable. En combinación con el interferona-alfa pegilada y ribavirina, el 90% de los pacientes demostraron este efecto. **Los resultados están adjuntos.** Estos datos claramente sustentan el nivel inventivo de la combinación reclamada.

Adicionalmente, para sustentar el nivel inventivo se adjunta con este escrito **un documento publicado en julio de 2012 que sustenta la patentabilidad** de las combinaciones reclamadas. En la Figura 1 de la página 5233 se muestra que la combinación del tratamiento con daclatasvir (DCV) y asunaprevir (ASV) reduce el surgimiento de colonias resistentes. Específicamente, la figura 1a muestra las células incubadas por 4 semanas con una combinación de DCV y ASV en combinación junto con estas conteniendo solamente un agente u otro agente. Se puede observar que el surgimiento de resistencia se reduce significativamente (tal como se muestra en las placas en la esquina inferior derecha) cuando los dos compuestos se administran juntos con relación a cuando cada agente se administra solo. No había expectativa de éxito de que esta combinación particular mostrara estos resultados haciéndola de esta forma inventiva. El éxito limitado hasta el momento de otras combinaciones (mi representada es la primera en presentar para todas las combinaciones orales, basado en terapia sin-IFN, sin-RBV) lo que demuestra además el nivel inventivo de esta combinación en particular.

En conexión con lo anterior, traigo a colación lo expuesto por la Guía ¹ para Examen de solicitudes de patente expedida por la Superintendencia de Industria y Comercio, en la página 126, numeral 9.5.2 relacionada con la información complementaria y ejemplos comparativos para sustentar el nivel inventivo:

"Ante una objeción por falta de nivel inventivo el solicitante puede:

presentar argumentos o documentos para demostrar que había prejuicio técnico que conducía al experto en la materia contrario a la invención, o aportar pruebas, tales como ensayos comparativos, que demuestran la presencia de un efecto técnico sorprendente o una ventaja de la invención respecto al estado de la técnica cercano.."

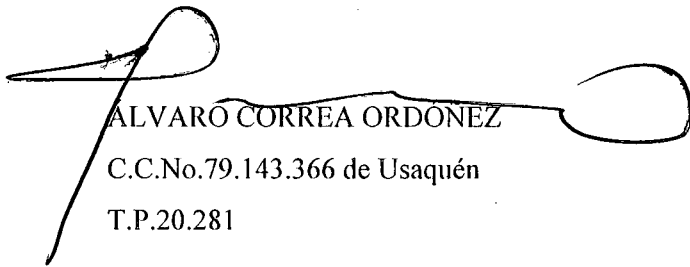
¹ Guía para examen de solicitudes de patente de invención y modelo de utilidad, SIC, mayo de 2011.

Solicitamos respetuosamente al examinador revisar los datos y publicación que se presenta con este escrito como sustento del nivel inventivo de la solicitud en estudio.

Teniendo en cuenta los argumentos arriba expuestos así como los datos comparativos y publicación que sustenta la patentabilidad de esta invención, se puede concluir que la solicitud que la invención propuesta por el solicitante cumple con los requisitos de patentabilidad y de esta manera es susceptible de ostentar el título de patente de invención.

En virtud de lo anterior, solicito que se acepten las razones expuestas en el presente documento y en consecuencia, se otorgue el privilegio de patente de invención para el invento que se tramita en este expediente.

Atentamente,



ALVARO CORREA ORDONEZ
C.C.No.79.143.366 de Usaquén
T.P.20.281

Anexos: - Datos de prueba que sustenta nivel inventivo
 - Publicación de julio de 2012 que sustenta patentabilidad

BOGMGT/BOGDMS-#2058832-V1-P2012_000017-MEMORIAL_RESPUESTA_AUTO_DE_FONDO.DOC



	<i>Group A: NS5A Inhibitor and NS3 Protease Inhibitor (n=11)</i>	<i>Group B: NS5A Inhibitor, NS3 Protease Inhibitor, Pegylated Interferon Alpha and Ribarvirin (n=10)</i>
Genotype 1a (n)	9	9
Median Baseline HCV RNA IU/mL	6.9 log ₁₀	6.7 log ₁₀
Median HCV RNA Decline Week 2 IU/mL	-5.1 log ₁₀	-5.3 log ₁₀
RVR* n(%)	7 (63.6%)	6 (60%)
eRVR* n(%)	4 (36.4%)	6 (60%)
cRVR* n(%)	5 (45.5%)	9 (90%)**

RVR (rapid virologic response) = undetectable HCV RNA at Week 4 of treatment
eRVR (extended rapid virologic response) = undetectable HCV RNA at both Week 4 and Week 12 of treatment
cRVR (complete rapid virologic response) = undetectable HCV RNA at Week 12 of treatment
*Intent-to-treat analysis; breakthrough = failure
** One subject with HCV RNA <25 IU/mL at Week 12, undetectable (UD, <10 IU/mL) on retesting.

The antiviral activity of the NS5A inhibitor (formula (I); 60 mg QD) and the NS3 protease inhibitor (formula (II); 600 mg BID) alone (Group A) or with pegylated interferon alpha and ribavirin (Group B) for 24 weeks in HCV genotype 1 null responders. The primary aim was to determine the proportion of subjects achieving undetectable HCV RNA (<10 IU/mL) at Weeks 2 and 4 of therapy and 24 weeks post-treatment. A Week 12 interim analysis was performed. Twenty-one patients (11 Group A, 10 Group B) were randomized in a sentinel cohort. The median age was 55 years; 13 were male and 16 were white.

As shown in Table 2, six (54.5%) subjects experienced viral breakthrough while all subjects in Group B maintained viral suppression. Viral breakthrough occurred exclusively in individuals infected with genotype 1a, occurring as early as Week 3 and as late as Week 12. The two genotype 1b subjects in Group A remained HCV RNA undetectable. The six subjects with breakthrough received additional pegylated interferon alpha and ribavirin. HCV RNA fell to undetectable in two subjects and to <25 IU/mL in another two subjects while the other two had ≥ 1.5 log₁₀ decreases in HCV RNA. No deaths,

significant adverse events, or discontinuations due to adverse events were recorded during the analysis period. Diarrhea was the most common adverse event and was mainly mild to moderate in severity.

Antimicrobial Agents
and Chemotherapy

**Effect on Hepatitis C Virus Replication of
Combinations of Direct-Acting Antivirals,
Including NS5A Inhibitor Daclatasvir**

Lenore A. Pelosi, Stacey Voss, Mengping Liu, Min Gao and
Julie A. Lemm
Antimicrob. Agents Chemother. 2012, 56(10):5230. DOI:
10.1128/AAC.01209-12.
Published Ahead of Print 30 July 2012.

Updated information and services can be found at:
<http://aac.asm.org/content/56/10/5230>

SUPPLEMENTAL MATERIAL	<i>These include:</i> http://aac.asm.org/content/suppl/2012/09/10/AAC.01209-12.DC Supplemental.html
REFERENCES	This article cites 62 articles, 27 of which can be accessed free at: http://aac.asm.org/content/56/10/5230#ref-list-1
CONTENT ALERTS	Receive: RSS Feeds, eTOCs, free email alerts (when new articles cite this article), more»

Information about commercial reprint orders: <http://journals.asm.org/site/misc/reprints.xhtml>
To subscribe to to another ASM Journal go to: <http://journals.asm.org/site/subscriptions/>



Effect on Hepatitis C Virus Replication of Combinations of Direct-Acting Antivirals, Including NS5A Inhibitor Daclatasvir

Lenore A. Pelosi, Stacey Voss, Mengping Liu, Min Gao, and Julie A. Lemm

Department of Virology, Bristol-Myers Squibb Research & Development, Wallingford, Connecticut, USA

Three hepatitis C virus (HCV) inhibitors, asunaprevir (ASV; BMS-650032), daclatasvir (DCV; BMS-790052), and BMS-791325, each targeting a different nonstructural protein of the virus (NS3, NS5A, and NS5B, respectively), have independently demonstrated encouraging preclinical profiles and are currently undergoing clinical evaluation. Since drug-resistant variants have rapidly developed in response to monotherapy with almost all direct-acting antiviral agents (DAAs) for HCV, the need for combination therapies to effectively eradicate the virus from infected patients is clear. These studies demonstrated the additive-synergistic effects on replicon inhibition and clearance of combining NS3 protease or NS5B RNA polymerase inhibitors with the first-in-class, NS5A replication complex inhibitor daclatasvir (DCV) and reveal new resistance pathways for combinations of two small-molecule inhibitors that differ from those that develop during monotherapy. The results suggest that under a specific selective pressure, a balance must be reached in the fitness costs of substitutions in one target gene when substitutions are also present in another target gene. Further synergies and additional novel resistance substitutions were observed during triple-combination treatment relative to dual-drug therapy, indicating that, in combination, HCV inhibitors can exert cross-target influences on resistance development. Enhanced synergies in replicon inhibition and a reduced frequency of resistance together lend strong support to the utility of combinations of DAAs for the treatment of HCV, and the identification of altered resistance profiles during combination treatment provides useful information for monitoring resistance in the clinic.

Hepatitis C virus (HCV) is a positive-stranded RNA virus in the *Flaviviridae* family of enveloped virions which affects an estimated 170 million people worldwide and is the major cause of chronic hepatitis. Currently, approximately 50% of patients infected with genotype 1 (gt 1), the most prevalent form of the virus, fail to achieve a sustained reduction in viral load with therapy employing pegylated alpha interferon (IFN- α) plus ribavirin (alfa/RBV) (52, 54, 56). A substantial fraction (20%) of chronically infected patients develop serious progressive liver disease, including cirrhosis or hepatocellular carcinoma. alfa/RBV treatment is associated with a high incidence (>30%) of adverse effects, some of which are of sufficient severity to cause patients to discontinue therapy (56). Despite the recent approval of two new direct-acting antiviral agents (DAAs), boceprevir and telaprevir, for use in combination with alfa/RBV (18, 47), their use may be limited by poor efficacy in some patient populations, inconvenient 3-times-daily dosing of the DAA, and association with side effects, including anemia, rash, and gastrointestinal effects, in addition to the well-documented spectrum of adverse effects associated with alfa/RBV. Although addition of these DAAs to the standard of care for HCV represents a significant improvement in patient therapy, there is still an unmet medical need for new agents and more-tolerable treatment regimens for newly diagnosed patients and those failing current therapies.

The 9.6-kb HCV genome encodes a polyprotein of about 3,000 amino acids via translation of a single, uninterrupted open reading frame. The polyprotein is cleaved co- and posttranslationally in infected cells by cellular and virus-encoded proteases to produce a multicomponent replication complex (8, 33). The serine protease encoded by the N-terminal region of NS3 is thought to be responsible for all downstream *cis* and *trans* proteolytic cleavages (9, 17). NS5A possesses no known enzymatic activity, but exists in different states of phosphorylation, and influences multiple functions at various stages of the viral replication cycle (41, 58). It has

been shown to interact with an extensive array of host proteins and to play a role in IFN resistance (37, 40). NS5B is the RNA-dependent RNA polymerase responsible for replication of HCV RNA (1, 4).

The essential roles of nonstructural proteins NS3 to NS5 in viral replication render each an attractive target for antiviral intervention (2). Clinical proof of concept has been achieved for a number of DAAs targeting some of these proteins, including the serine protease activity of NS3 (11, 16, 25, 31, 32, 45, 53) and the RNA-dependent RNA polymerase activity of NS5B (20, 26, 46; H. Tatum et al., poster 1163, presented at the 47th European Association for the Study of the Liver [EASL] Congress, Barcelona, Spain, 18 to 22 April 2011). More recently, daclatasvir (DCV) (Table 1) was the first NS5A replication complex inhibitor to show proof of concept in the clinic, demonstrating in early clinical testing the potential for this class of inhibitor to become a valuable component of an all-oral treatment regimen for HCV (15).

The high turnover rate and error-prone nature of the HCV RNA polymerase contribute to the production of potentially resistant viral quasiespecies. In practice, resistance has emerged to all small-molecule inhibitors of HCV tested as monotherapy except some nucleoside-nucleotide NS5B inhibitors. Resistance mutations have been identified both *in vitro* and *in vivo* upon treatment with nearly all inhibitors of HCV serine protease, NS5A, or allo-

Received 11 June 2012 Returned for modification 3 July 2012

Accepted 18 July 2012

Published ahead of print 30 July 2012

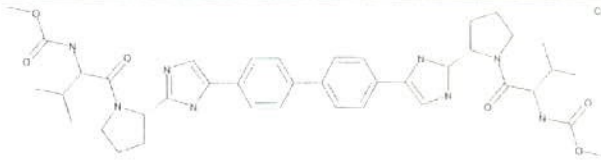
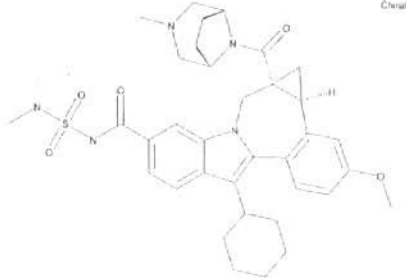
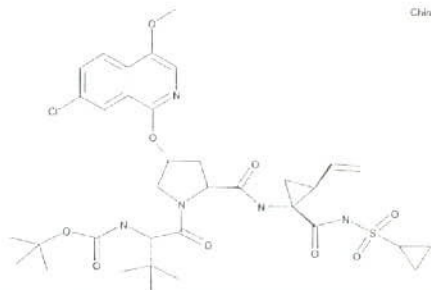
Address correspondence to Lenore A. Pelosi, lenore.pelosi@bms.com, or Julie A. Lemm, julie.lemm@bms.com.

Supplemental material for this article may be found at <http://aac.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/AAC.01209-12

TABLE 1 *In vitro* antiviral profiles of BMS HCV inhibitors^a

HCV inhibitor	Inhibitor structure	Value for indicated HCV replicon genotype			Therapeutic index (CC ₅₀ /EC ₅₀)
		1a (H77c) FC ₅₀	1b (Con1) EC ₅₀	1b (Con1) CC ₅₀	
Daclatasvir		50 ± 13 pM	9 ± 4 pM	17 ± 1 μM	1,900,000
BMS-791325		3 ± 1 nM	7 ± 2 nM	20 ± 6 μM	2,900
Asunaprevir		4 ± 0.8 nM	3 ± 2 nM	26 ± 5 μM	8,700

^a Data shown represent results of ≥3 independent tests ± standard deviations.

steric RNA polymerase inhibitors advanced to date (3, 19, 22, 23, 24, 30, 36, 38, 51, 52, 57, 60, 62), with good correlation observed between resistance emergence in the replicon system and *in vivo*. Recent literature indicates that treatment with combinations of non-cross-resistant inhibitors not only improves antiviral activity during treatment but also suppresses the posttreatment viral rebound often associated with monotherapy (19, 21, 26). To achieve a sustained viral response (SVR), it would be essential to use combination therapies similar to those that have recently been explored in replicon studies (5, 10, 29), animal models (44), and patient studies (14, 51) as a viable approach to improving the efficacy, tolerability, and compliance issues associated with current therapies. For this report, the effects of a combination approach to HCV therapy have been studied in the HCV replicon system using two- and three-drug combinations that include an NS5A replication complex inhibitor (DCV), the NS3 protease inhibitor asunaprevir (ASV), and the nonnucleoside NS5B RNA polymerase inhibitor BMS-791325.

MATERIALS AND METHODS

Cell lines. Bovine viral diarrhea virus (BVDV) and HCV replicon cell lines were previously described (27, 43) and were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 10% fetal bovine serum (FBS), and penicillin-streptomycin, with or without 0.3 to 0.5 mg/ml Geneticin (G418).

Efficiency of replicon clearance from cultured cells. HCV replicon cells (6 × 10⁴ per well in 6-well plates) and BVDV replicon cells (4 × 10⁴ per well) were treated with various 50% effective concentrations (EC₅₀s) of inhibitors in cell growth media for 1 week. After 7 days, media containing inhibitor were removed and cells were maintained in growth media containing 0.5 mg/ml G418. Media were changed twice weekly for a period of ~4 weeks. Plates were washed, and colonies were counted after cells were stained with 0.2% crystal violet. All conditions were tested in duplicate and repeated in separate experiments.

Selection of populations with reduced susceptibility to HCV inhibitors. HCV replicon cells were plated at a density of 6 × 10⁴ per 60-mm-diameter plate and maintained in growth media with 0.3 mg/ml G418 and various concentrations of inhibitor(s). BVDV replicon cells, plated at 2 × 10⁴ per 60-mm-diameter plate, were maintained in growth media containing 0.5 mg/ml G418 and treated in parallel with the same inhibitors. Fresh media containing compound were added every 3 to 5 days for a total of 4 weeks, after which plates were washed and cells were stained with 0.2% crystal violet or further selected for testing. All concentrations were tested in duplicate, and selections were repeated in separate experiments. gt 1b cultures undergoing triple-combination treatment required gradual (1.5- to 2-fold) ramping of drug concentrations, starting with the 5×-selected population, allowing 4 weeks incubation for each escalation in concentration in order to select high-level resistance.

HCV replicon luciferase and FRET assays. To evaluate compound efficacy, HCV replicon cells were incubated in 96-well plates in the presence of compound for 3 days. For replicons containing a luciferase re-

porter gene, Renilla luciferase activity was then assayed using a Renilla Luciferase Assay System or DualGlo Luciferase Assay System (Promega Corporation, Madison, WI), according to the manufacturer's directions. Plates were read on a TopCount NXT Microplate Scintillation and Luminescence Counter (Packard Instrument Company, Meriden, CT). For replicons lacking a reporter gene, a fluorescence resonance energy transfer (FRET) assay monitored NS3 protease activity as a measure of HCV replicon levels (43). The 50% effective concentration (EC₅₀) was calculated using the four-parameter logistic formula $Y = A + [(B - A)/(1 + (C/x)^D)]$, where A and B denote minimal and maximal percent inhibition, respectively, C represents the EC₅₀, D represents the hill slope, and x represents the compound concentration.

Cell-based inhibitor combination assays. For combination studies, each inhibitor was tested at 11 concentrations. The compounds were tested as monotherapies and in combinations at various concentration ratios. Cells were exposed to compounds for 3 days, and the amount of HCV inhibition was then determined using the Dual-Glo luciferase assay as described above. The potential cytotoxicities of these combined agents were also analyzed in parallel by alamarBlue staining. The 50% cytotoxic concentration (CC₅₀) values were calculated using the four-parameter logistic formula described above.

The degree of antagonism, additivity, or synergy was determined from combination dose-response curves which were fit to assess the antiviral effects of the drug treatment combinations. The concentration ratios were analyzed using the method of Chou (6). All estimates were computed using SAS Proc NLIN biostatistical software and a four-parameter logistic. Combination indices were tested for departure from additivity using isobologram methods. Asymptotic confidence intervals were also calculated for each of the combination indices. These intervals are used to test for departure from additivity by comparing the bounds to a value of 1—a lower bound of the interval greater than 1 indicates antagonism, an upper bound of less than 1 indicates synergism, and a value of 1 contained in the interval indicates additivity.

Identification of mutations selected in resistant populations. RNA was isolated from populations of resistant cells using either TRIzol or an RNeasy 96 kit (Qiagen Inc., Valencia, CA) in accordance with the manufacturer's directions. First-strand cDNA synthesis was performed using 1 to 3 µg of total RNA and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) primed with gene-specific oligonucleotide primers. PCR was performed using the cDNA and pairs of primers flanking the genes of interest (NS3 to NS5B). PCR products were sequenced, and mutations were identified relative to vehicle-treated populations. PCR products were purified and cloned using TOPO PCR cloning methods (Invitrogen, Carlsbad, CA). DNA was sequenced from ~100 clones to establish the frequency and coincidence of mutations in a population.

To generate mutant replicons, point mutations were generated with a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, and mutations were confirmed by sequencing.

Transient replication assays. Replicon clones were linearized with ScaI and transcribed *in vitro* using an Ambion T7 MegaScript kit (Ambion, Austin, TX) or a T7 RiboMAX Express Large-Scale RNA Production System (Promega, Madison, WI) according to manufacturer's directions. Transcribed RNA (3 to 5 µg) was transfected into cured Huh-7 cells (~2 × 10⁶ in 60-mm-diameter dishes) with DMRIE-C reagent (Invitrogen Corporation, Carlsbad, CA) following the manufacturer's protocol. After 4 to 6 h, transfected cells were transferred to 96-well assay plates (10⁴ cells/well) and incubated in the presence of inhibitors for 72 h. Renilla luciferase assays were performed as described above.

RESULTS

Inhibition of replicon with combinations of HCV inhibitors. The HCV replicon system is widely acknowledged as a predictive tool for the development of DAAs that target HCV RNA replication (2). In the gt 1 HCV replicon system, DCV, ASV, and BMS-

TABLE 2 Combination of daclatasvir with asunaprevir or BMS-791325

Expt	Ratio of inhibitor to daclatasvir in paired combinations	Overall result(s) for indicated combination		
		Asunaprevir + daclatasvir	BMS-791325 + daclatasvir	Asunaprevir + BMS-791325 + daclatasvir (1:1:1)
1	1:1	Synergy/additivity	Synergy	Additivity
	2.5:1	Synergy	Additivity	
	1:2.5	Synergy	Synergy	
2	1:1	Additivity	Synergy/additivity	Additivity
	2.5:1	Synergy/additivity	Additivity	
	1:2.5	Synergy/additivity	Synergy	
3	1:1	Synergy	Synergy/additivity	Additivity
	2.5:1	Additivity	Synergy/additivity	
	1:2.5	Synergy	Synergy	

791325 exhibit picomolar to low-nanomolar potency (Table 1). The virology profile and clinical effects of DCV (15, 42, 45), ASV (37, 38), and BMS-791325, a nonnucleoside allosteric inhibitor of NS5B that binds a site on the surface of the thumb domain of the RNA-dependent RNA polymerase (Tatum et al., poster 1163; J. E. Kadow et al., presented at the American Chemical Society Spring 2012 National Meeting and Exposition, San Diego, CA, 25 to 29 March, 2012; Gentles et al., unpublished data), have been reported. These inhibitors were used to investigate the spectrum of events resulting from simultaneously targeting multiple essential proteins of HCV *in vitro*. Of particular interest was determining the effect of the NS5A replication complex inhibitor DCV in combination with other DAAs.

We used the method of Chou (6) to evaluate the additive, antagonistic, or synergistic effects of combination therapies on HCV inhibition in a 3-day replicon assay. Table 2 shows the results of testing DCV and other inhibitors as monotherapies or in combination in HCV replicon experiments. The CC₅₀s of these combined agents, analyzed in parallel by alamarBlue staining, were greater than the highest tested inhibitor concentration. The effects of DCV in combination with ASV or BMS-791325 indicate mixed additivity and/or synergy over a range of molar ratios of inhibitors. When the NS5A, NS5B, and NS3 inhibitors were tested in a 3-drug combination, additive effects were observed at all effective doses. Taken as a whole, the results from multiple experiments demonstrate that combinations of these inhibitors display mixed additivity and/or synergy at the 50%, 75%, and 90% effective dose levels of drug. Importantly, no antagonistic effects were observed with any of these combinations and no increase in cytotoxicity was observed.

Resistance selection with combinations of HCV inhibitors. Upon prolonged exposure of replicon cells to HCV inhibitors, colonies develop as resistant variants are selected. Figure 1 shows the relative densities of resistant colonies under conditions in which DCV was combined with either ASV (panel a) or BMS-791325 (panel b) in cultures examined after continuous 4-week DAA exposure in the presence of G418. In multiple experiments, populations of resistant colonies were markedly reduced when cells were treated with a combination of DCV and ASV (Fig. 1a) or of DCV and BMS-791325 (Fig. 1b) compared with resistant pop-

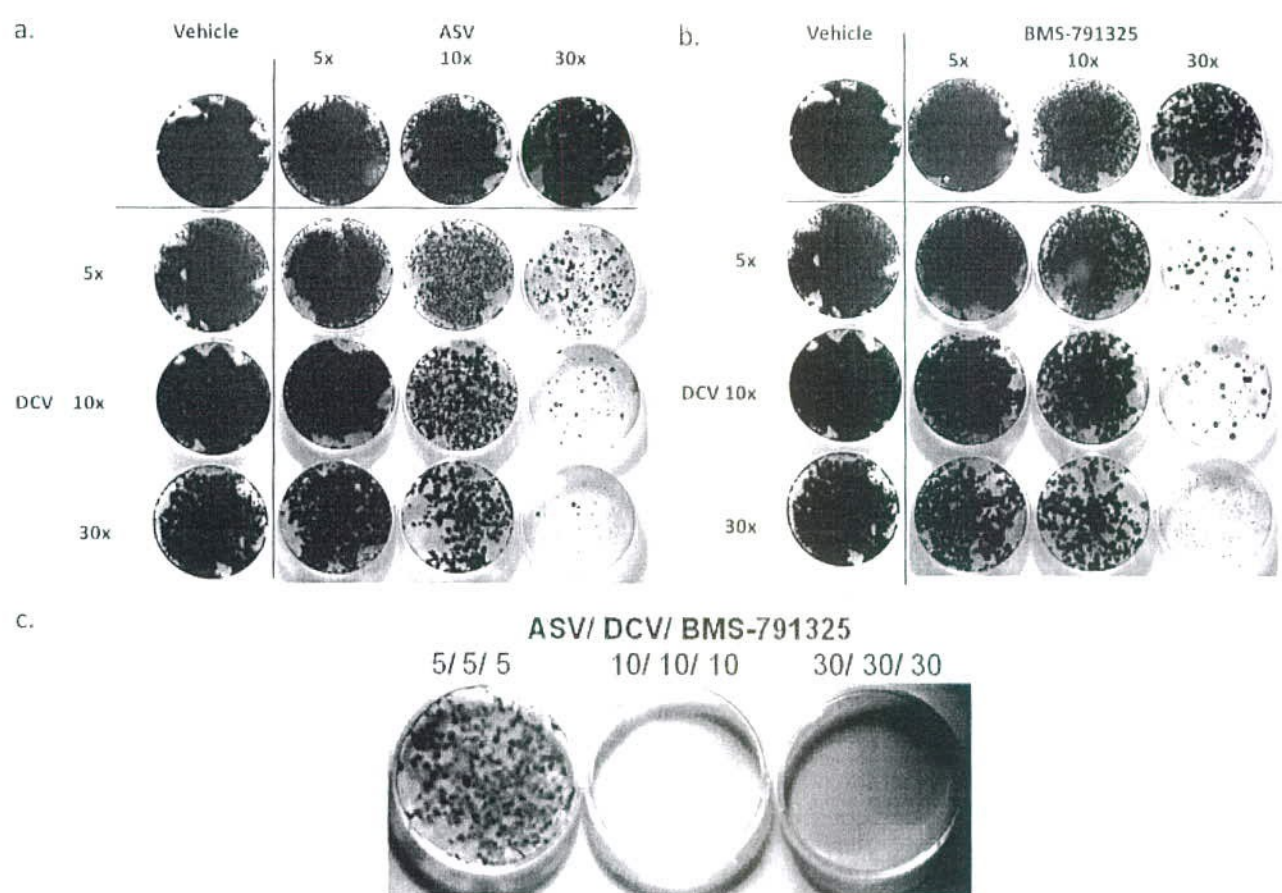


FIG 1 Combination treatment reduces the emergence of resistant colonies. gt 1b HCV replicon cells were incubated for 4 weeks with BMS-790052 (DCV), BMS-791325, or BMS-650032 (ASV) as monotherapy and dual therapy (a and b) and triple therapy (c) at 5 $\times$, 10 $\times$, and 30 $\times$ EC₅₀. Colonies were visualized by crystal violet staining. Data shown are representative of the results of three independent experiments.

ulations resulting from treatment with similar EC₅₀ multiples of the DAAs alone. BVDV replicon cells treated in parallel showed no reduction in colony formation, suggesting a specific anti-HCV effect (data not shown). Interestingly, fewer resistant colonies were observed with combinations composed of 5 $\times$ the EC₅₀ of DCV with any concentration of NS5B or NS3 inhibitor than were seen with alternate combinations pairing 5 $\times$ the EC₅₀ of either the NS5B or NS3 inhibitor with any concentration of DCV (Fig. 1a and b). For example, although both plates were exposed to a total of 35 $\times$ the EC₅₀ of DAA, the plate combining 5 $\times$ DCV with 30 $\times$ BMS-791325 developed far fewer resistant colonies than the plate combining 30 $\times$ DCV with 5 $\times$ BMS-791325 (Fig. 1b). A similar phenomenon was observed for DCV in combinations with ASV (Fig. 1a).

Triple-DAA treatment of HCV replicon cells with inhibitors at 10 $\times$ and 30 $\times$ their respective EC₅₀s resulted in complete clearance of HCV replicon (Fig. 1c) while having no effect on BVDV replicon cells (not shown). In the presence of 5 $\times$ the EC₅₀ of the three DAAs (total, 15 EC₅₀ multiples), fewer colonies were observed than after treatment with a 30 $\times$ concentration of any single DAA or many pairs of DAAs. Similarly, in the gt 1a replicon, combinations of two or three DAAs more effectively reduced resistant colony formation than any single DAA at similar EC₅₀ mul-

tiples. The overall suppressive effect in gt 1a was slightly reduced compared to that in gt 1b (not shown).

Replicon clearance studies were also performed with the DAAs in the absence of G418 selection to evaluate the ability of various inhibitor combinations to eradicate HCV replicon RNA from the cells. Similar to the resistance studies, replicon clearance occurred more efficiently in gt 1b than gt 1a. In both gt 1a and gt 1b, low EC₅₀ multiples of DCV effected a much greater reduction in resistant-colony formation than the same multiple of the other DAAs and a greater-than-additive effect on replicon clearance was observed using combinations of DAAs. Overall clearance with the DAA dual combinations in gt 1a was reduced 2- to 4-fold compared to gt 1b at 3 $\times$ and 9 $\times$ the EC₅₀; the difference was greater (6- to 17-fold) at 27 $\times$ the EC₅₀.

Genotypic and phenotypic analysis of resistant variants. Resistant variants identified from selection with each DAA inhibitor class used in these studies have been previously described (12, 13, 27, 29, 30, 38, 45, 52, 61). The major gt 1b-resistant substitutions are at residues 168 for NS3, 31 and 93 for NS5A, and 495 for NS5B, while the major gt 1a-resistant substitutions are at residues 155 for NS3, 28, 30, 31, and 93 for NS5A and 495 for NS5B. To study the impact of different DAA combinations on the emergence of resistance, and in an attempt to use these HCV-specific inhibitors as a

TABLE 3 Characterization of gt 1b variants identified in dual selections using transient replication assays

Selection condition	Variant ^a			Fold resistance ^b			Replication efficiency relative to WT level
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	325 ^c	
5× or 30× ASV	D168V	—	—	>100	0.9	2	0.3–0.5
5× DCV	—	L31M	—	1	2–5	1	0.5–1.5
30× DCV	—	Y93H	—	1	10–33	1	0.1–0.3
	—	L31M-Y93H	—	ND ^d	4,200 ^e	ND	0.4
5× or 20× 325	—	—	P495A	1	1	9–16	0.3–0.8
20× 325	—	—	P495S	1	1	40–86	0.01–0.1
5× ASV/DCV	N77S	R30Q-L31M	—	5–11	6–16	1	0.5–1.2
30× ASV/DCV	N77S-R155Q	R30Q-L31M-Y93H	—	80–210	31,000–37,000	1	0.2–0.5
	N77S-R155Q	R30H-L31M	—	62–170	150–330	1	0.3–0.7
5× DCV/325	—	R30Q-L31M	P495A	1	12–22	9–20	0.4–0.8
20× DCV/325	—	R30Q-L31F	P495S	1	46–62	111–128	0.1
Site-directed mutant	N77S	—	—	3–9	1	1	0.2–0.5
Site-directed mutant	R155Q	—	—	8–9	1	1	0.01–0.02
Site-directed mutant	N77S-R155Q	—	—	93–113	1	1	0.02–0.1
Site-directed mutant	—	R30Q	—	1	2	1	0.7–1.1
Site-directed mutant	—	R30H	—	1	2–8	1	0.1–0.2
Site-directed mutant	—	R30Q-L31M	—	1	4–16	1	0.9–1.2
Site-directed mutant	—	R30Q-L31F	—	2	57–85	1	1.0–1.2
Site-directed mutant	—	R30Q-L31M-Y93H	—	1	25,000–51,000	1	0.7–1.4

^a Replicons containing specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variant constructs are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant that includes any linked mutations. —, WT sequence.

^b Fold resistance was determined by dividing the mutant HCV replicon EC₅₀ by the WT replicon EC₅₀. WT EC₅₀s were 2.0 ± 0.6, 0.002 ± 0.001, and 8.3 ± 2.2 nM for ASV, DCV, and BMS-791325, respectively. Results represent the ranges of values determined from two or three independent experiments.

^c 325, BMS-791325.

^d ND, not determined.

^e Value was taken from the work of Fridell et al. (12).

tool to study potential interactions of viral proteins, this report emphasizes only the genotypic and phenotypic analysis of unique or less frequently observed substitutions identified from dual- and triple-DAA selections. Resistant variants selected with one, two, and three DAAs in HCV 1a and 1b replicons were analyzed by both population and clonal sequencing to determine the percentage and linkage of mutations within and between genes in the population. Substitutions observed with a frequency of >10% were engineered into wild-type (WT) replicon to evaluate the contribution of the altered residue to resistance and impact on replication fitness in transient replication assays.

Analysis of NS5A and NS3 dually resistant populations. Similar to previous reports, the major gt 1b resistance substitutions selected by DCV and ASV were L31M and Y93H in NS5A (12, 27) and D168V in NS3 (38, 45) (Table 3), respectively. For the dual selection performed in parallel, the most striking substitution was N77S in NS3. The N77S variant in gt 1b under dual selection conditions with boceprevir and a 2-C-methyl-adenosine NS5B inhibitor has been previously reported (5). In that case, N77S exhibited no phenotype, as it conferred no resistance to boceprevir and did not affect replication efficiency. However, in this case, N77S conferred a low level (3- to 9-fold) of resistance to ASV by itself and, in combination with R155Q, which was itself associated with 8- to 9-fold ASV resistance, demonstrated an amplified level of resistance of 93- to 113-fold in the dually substituted N77S-R155Q variant. In NS5A, R30Q/H linked with L31M emerged in the dual selection. R30H, which has not been reported before, conferred minimal (2- to 8-fold) resistance to DCV but displayed

significant (150- to 330-fold) resistance in combination with L31M. When linkage of N77S-R155Q in NS3 occurred with multiple substitutions in NS5A (R30Q-L31M-Y93H), very high levels of resistance to both DCV (31,000- to 37,000-fold) and ASV (80- to 210-fold) were observed. The observation of the N77S substitution in NS3 only during dual selection was unexpected and indicates the influence of the NS5A inhibitor on the emergence of NS3 resistance *in vitro*. In gt 1a replicon cells, R155K was selected by ASV, whereas a D168G substitution was observed also during dual selection with ASV and DCV, although substitutions at R155 and D168 in NS3 were not linked (Table 4). Selection with DCV yielded two predominant NS5A substitutions, Q30H and K68R, while in the dual selection, an additional NS5A resistance substitution, M28T, was also observed. The novel arginine substitution at residue 68 of NS5A did not confer any resistance but appeared to increase the relative fitness of the 1a variant (1 to 4 times the efficiency of the WT), whereas replication of the Q30H-K68R mutant was greatly impaired. A dramatic improvement was observed with the dually selected D168G/Q30H-K68R clone, which replicated at 30% to 70% of the level of the WT whereas each of the NS3 and NS5A mutants alone had extremely impaired replication efficiency (4% to 9% the level of the WT), suggesting an NS3-NS5A interaction that restored replicase activity. Interestingly, combination of the Q30H-K68R substitutions in NS5A with D168G in NS3 reduced the level of NS5A resistance.

Analysis of NS5A-NS5B dually resistant populations. gt 1b replicon cells treated with DCV and BMS-791325 alone or in combination yielded resistant populations with substitutions at

TABLE 4 Characterization of gt 1a variants identified in the dual selections using transient replication assays

Selection condition	Variant ^a			Fold resistance ^b			Replication efficiency relative to WT level
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	325 ^c	
10× or 30× ASV	R155K	—	—	17–48	1	1	0.3–0.5
10× or 30× DCV	—	Q30H-K68R	—	1	1,280–2,000	3	0.04–0.07
10× or 30× 325	—	—	A421V	1	1	1–3	0.3–0.9
	—	—	P495L	2	1	88–100	0.07–0.1
30× DCV/ASV	R155K	Q30H-K68R	—	46–68	667–700	1	0.5–0.8
	R155K	M28T-K68R	—	34–61	1,125–13,333	1	0.4–1
	D168G	Q30H-K68R	—	18–30	111–333	1	0.3–0.7
30× DCV/325	—	Q30H-K68R	A421V	1	1000	2	0.8–1
	—	Q30H-K68R	P495L	2	1600	61–84	0.03–0.07
	—	Q30H-K68R	L392I	2	2200	6–16	0.06–0.19
Site-directed mutant	D168G	—	—	8–16	1	1	0.07–0.09
Site-directed mutant	—	M28T	—	1	750–820	1	0.2–0.4
Site-directed mutant	—	Q30H	—	1	1111–1967	1	0.4–0.6
Site-directed mutant	—	K68R	—	1	1	1	1–4.6
Site-directed mutant	—	—	L392I	1	1	5–7	0.1–0.4

^a Replicons containing specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variant constructs are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant that includes any linked mutations. —, WT sequence.
^b Fold resistance was determined by dividing the mutant HCV replicon EC₅₀ by the WT replicon EC₅₀. WT EC₅₀s were 0.7 ± 0.3, 0.006 ± 0.002, and 2.2 ± 0.9 nM for ASV, DCV, and BMS-791325, respectively. Results represent the range of values determined from two or three independent experiments.
^c 325, BMS-791325.

known positions in NS5A (R30, L31, Y93) (12) and NS5B (P495) (J. A. Lemm et al., unpublished data) (Table 3). For dual selection with NS5A and NS5B inhibitors, L31M and L31F were linked with R30Q (Table 3) whereas only L31M was selected by DCV alone. Phenotypic analysis of R30Q-L31F, with or without NS5B substitution P495S, revealed much greater (46- to 85-fold) resistance to DCV than the single L31F (5-fold) (12) and R30Q (Table 3) substitutions. While the NS5A R30Q-L31F variant replicated as efficiently as the WT, linkage with the NS5B P495S substitution greatly impaired replication (Table 3).

In gt 1a, selection with BMS-791325 yielded substitutions of A421V and P495L in NS5B whereas, as stated earlier, DCV selection generated the Q30H-K68R variant in NS5A (Table 4). In dual selections, an additional L392I substitution in NS5B was also observed. Clonal analysis of the dual selections revealed linkage of Q30H-K68R in NS5A with each of the single NS5B substitutions (L392I, A421V, P495L), as no two NS5B changes were found in the same clone. The A421V NS5B substitution conferred no resistance to BMS-791325; however, it did increase the replication efficiency of the Q30H-K68R NS5A variant 10- to 15-fold (Table 4). The L392I substitution in NS5B has not been reported for gt 1a, although it was previously shown in gt 1b to confer low-level resistance (15- to 20-fold) to an indole-*N*-acetamide nonnucleoside inhibitor (50). In gt 1a, L392I conferred low-level (5- to 16-fold) resistance to BMS-791325 while the P495L variant gave the expected high-level (60- to 100-fold) resistance.

Triple selection with NS5A, NS5B, and NS3 inhibitors. gt 1b cells treated with a fixed concentration greater than 5× the EC₅₀ of DCV, ASV, and BMS-791325 together were unable to survive. However, resistant variants emerged when cells were subjected to serial passage with gradually increasing concentrations of the three inhibitors. Populations treated in this way with up to 15× the EC₅₀ selection consisted of an interesting mix of previously

seen and new variants. Compared to the dual selections, a different set of NS3 substitutions, Q41R with Q80R and Q80R with R155Q, were selected during triple-DAA treatment. Presumed to be adaptive or compensatory changes, substitutions of glutamine residues at positions 41 and 80 in NS3 are well documented, and Q80R was selected with very low frequency in a gt 1b replicon upon treatment with ASV (38). ASV resistance to Q41R or Q80R was low but measurable (4- to 5-fold) and roughly additive (7- to 12-fold) under conditions in which both substitutions were present (Table 5). The combination of Q80R with R155Q conferred 30- to 100-fold-greater resistance to ASV, which was 4× to 10× higher than was observed with each NS3 mutation alone. Despite differences in the NS3 sites affected by the triple-DAA regimen, the sites of resistance in NS5A and NS5B during treatment with a triple-drug combination were the same as those targeted with dual-drug selection regimens: R30, L31, and Y93 in NS5A and P495 in NS5B (Table 5). Replication efficiency for these reconstituted triply resistant variants was significantly impaired.

Triple selection in the gt 1a replicon cells readily yielded a cell line that displayed significant resistance to all three inhibitors. Along with known substitutions at amino acids 155 and 168, several additional substitutions were identified in NS3, and clonal analysis revealed 100% linkage between NS3 D168E and M179T, R155K and M179A, and Q80R and Y134H, as well as between T389I and A421V in NS5B (Table 6). The Q89R, Y134H, and M179A/T NS3 mutations showed no resistance to ASV and did not significantly affect resistance levels in combination with other substitutions except M179A, which slightly (3- to 5-fold) enhanced R155K resistance in the context of the triple mutant (Table 6). Alone, the Q89R change significantly enhanced replication (2 to 6× the efficiency of the WT) and may play a similar role in the fitness of triple mutants to allow replication of these highly altered replicons. Along with substitutions at amino acid 495, a new com-

TABLE 5 Characterization of gt 1b variants identified in the triple selection using transient replication assays

Selection condition	Variant ^a			Fold resistance ^b			Replication efficiency relative to WT level
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	BMS-791325	
10× or 15× triple^c	Q80R-R155Q	R30Q-L31M	P495A	30–93	5–15	17–22	0.02–0.1
	Q80R-R155Q	R30Q-L31M-Y93H	P495A	86–99	>500	8–22	0.01
Site-directed mutant	Q41R	—	—	4	1	1	1.0–1.4
Site-directed mutant	Q80R	—	—	4–5	1	1	0.5–1.0
Site-directed mutant	Q41R-Q80R	—	—	7–12	1	1	1.0–1.2
Site-directed mutant	Q80R-R155Q	—	—	42–73	1	1	0.5–1.0

^a Replicons containing a specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variant constructs are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant including any linked mutations. —, WT sequence.

^b Fold resistance was determined by dividing the mutant HCV replicon EC₅₀ by the WT replicon EC₅₀. WT EC₅₀s were 2.0 ± 0.6, 0.002 ± 0.001, and 8.3 ± 2.2 nM for ASV, DCV, and BMS-791325, respectively. Results represent the range of values determined from two or three independent experiments.

^c Selection of viable triple mutants in gt 1b required sequential passage in gradually increasing concentrations of inhibitor as detailed in Materials and Methods.

bination of NS5B mutations, T389I-A421V, that conferred low-level (3- to 8-fold) resistance to BMS-791325 was identified in the triple selection. It also appears that high-level resistance to NS3 and NS5B inhibitors was not selected in the same clone. High-level NS5B resistance due to the P495L substitution is associated with low-level NS3 resistance from Q80R, and, conversely, high-level NS3 resistance (Q89R-R155K-M179A) is linked to T389I-A421V changes in NS5B which confer low-level resistance. Levels of NS5A resistance remain consistent in all three triple mutants. Table 7 provides a summary of the resistant substitutions observed during treatment with combinations of DAAs in these studies. However, as reported here, additional replication-enhancing substitutions, particularly during triple-DAA treatment, were also observed.

DISCUSSION

Having shown promising antiviral activity in early clinical trials (15), the picomolar NS5A replication complex inhibitor DCV was examined in combination with other DAAs targeting distinct steps in the replication process. Since it has yet to be established

whether efficacy and resistance profiles for combinations of DAAs reflect the sum of the profiles of the individual drugs, *in vitro* replicon studies may provide insight into clinical outcomes for combinations of non-cross-resistant DAAs in terms of impact on viral replication and emergent resistance pathways.

In 72-h replicon assays, combinations of DCV with NS3 protease and/or NS5B polymerase inhibitors demonstrated additive to synergistic inhibitory effects on replicon activity. Replicon curing performed over a 7-day treatment period provided evidence of a similar, greater-than-additive effect from combining these inhibitors. Clearance from gt 1b replicons was generally twice as efficient as from gt 1a, a genotype difference that appears to extend to clinical outcomes for DCV (15, 34). Moreover, a low EC₅₀ multiple of DCV coupled with either the NS3 or NS5B-targeted DAA had a much more profound impact on limiting resistance development than did a low EC₅₀ multiple of either NS3 or NS5B inhibitor combined with DCV. The impressive dose-response curve for curing with DCV compared to the other inhibitors suggests that NS5A inhibition more readily attenuates the ability of HCV

TABLE 6 Characterization of gt 1a variants identified in the triple selection using transient replication assays

Selection condition	Variant ^a			Fold resistance ^b			Replication efficiency relative to WT level
	NS3	NS5A	NS5B	Asunaprevir (ASV)	Daclatasvir (DCV)	BMS-791325	
30× triple	Q80R-Y134H	Q30H-K68R	P495L	10–12	1,000	25–70	0.03–0.07
	Q89R-D168E-M179T	Q30H-K68R	A421V	15–35	1,500–2,200	2	0.05–0.12
	Q89R-R155K-M179A	Q30H-K68R	T389I-A421V	67–170	800–1,375	5–8	0.72–1.9
Site-directed mutant	—	Q30H-K68R	T389I-A421V	2	1,400	4–5	0.2–0.5
Site-directed mutant	Q80R	—	—	6–11	2	2	0.4–0.6
Site-directed mutant	Q89R	—	—	2	1	1	2.0–6
Site-directed mutant	Y134H	—	—	2	2	2–3	0.2–0.3
Site-directed mutant	D168E	—	—	47–74	1	1	0.6–1.2
Site-directed mutant	M179A	—	—	1	1	1	0.6–1.2
Site-directed mutant	M179T	—	—	1	1	1	0.3–0.7
Site-directed mutant	Q89R-R155K	—	—	26–67	2	1	0.4–1.1
Site-directed mutant	—	—	T389I-A421V	1	1	3–5	0.5–1.2
Site-directed mutant	—	—	T389I	1	1	1	1.1–2.6

^a Replicons containing specific substitution(s) identified from selections were tested for resistance and replication fitness. Major selected variants are in bold font. Site-directed mutants were not selected but were generated as controls in the WT backbone to assess impact on phenotype. For each selection condition, each row represents a unique variant that includes any linked mutations. —, WT sequence.

^b Fold resistance was determined by dividing the mutant HCV replicon EC₅₀ by the WT replicon EC₅₀. WT EC₅₀s were 0.7 ± 0.3, 0.006 ± 0.002, and 2.2 ± 0.9 nM for ASV, DCV, and BMS-791325, respectively. Results represent the range of values determined from two or three independent experiments.

TABLE 7 Summary of predominant resistance substitutions selected during inhibitor treatment

Target(s) of inhibitors used for selection	Resistance substitution(s)					
	Genotype 1b			Genotype 1a		
	NS3	NS5A	NS5B	NS3	NS5A	NS5B
NS3	D168V			R155K		
NS5A		L31M, Y93H			Q30H	
NS5B			P495A/S/L			P495L
NS3, NS5A	N77S, R155Q	R30Q/H, L31M, Y93H		R155K, D168N/G	Q30H, M28T	
NS5A, NS5B		R30Q/H, L31M/E, Y93H	P495A/S/L		Q30H	L392I, P495S/L
NS3, NS5A, NS5B	Q41R, Q80R, R155Q	R30Q/H, L31M, Y93H	P495A	Q80R, R155K, D168E	Q30H	P495L, T389I-A421V

replicons to survive. Since data suggest homotypic oligomeric interactions of the NS5A protein (7, 35, 59), it is possible that binding of a single NS5A inhibitor molecule may induce conformational changes that translate to adjacent NS5A molecules, cooperatively impacting the functionality of the entire replication complex. Higher concentrations of NS5A would not have such an impact, as the system may become saturated. Such a model could explain the steep dose-response curve of clearance with DCV and suggests an advantage of inclusion of DCV in DAA combination regimens.

It is known that minor changes in selection conditions can affect the pattern of resistance observed. Here we performed selections with single and multiple inhibitors in parallel to compare resistance emergence results between monotherapy and combination DAA treatment under the same selective conditions. In general, the resistant variants observed in this study were all at reported or predicted positions but were distinct in terms of frequency and emergence among different DAA combinations. In multiple experiments, the NS3 substitution N77S emerged in gt 1b replicons during two-DAA treatment. Substitution at this residue has been detected in gt 1a replicons selected with a close analog of ASV (39) and in gt 1b replicons selected with a combination of a protease inhibitor (boceprevir) and nucleoside analog NS5B inhibitor, without an apparent phenotype, but to our knowledge N77S has not previously been reported as the predominant resistance variant in either gt 1b or gt 1a with any protease inhibitor. This substitution is located at the end of the enzyme's EF loop and may interact with the bulky P2 moiety of inhibitors such as ASV. Although it is a low-level-resistance substitution, N77S may influence subsequent changes in the population, as it was observed first under conditions of low dual-selective pressure and was 98% linked to R155Q in populations selected under conditions of higher selective pressure with the two DAAs. In contrast, populations of cells treated with any level of ASV pressure alone were homogeneous for D168V. In these studies, the opposite was observed in gt 1a, where substitution at amino acid 168 occurred only during combination treatment whereas monotherapy elicited a R155K substitution. In NS5A, the mix of amino acid changes at positions 30, 31, and 93 differed in gt 1b populations emerging under conditions of single- and dual-drug selection. The new R30H substitution in gt 1b was observed only in the NS5A/NS3 dual selection, and while it showed minimal resistance itself, it enhanced resistance of L31M ~100-fold. In contrast, position 30 plays an important direct role in resistance in gt 1a NS5A, where the change from WT glutamine to histidine (Q30H) conferred significant (>1,000-fold) resistance to DCV, highlighting the genotype context-dependent role of this residue. In general,

resistance in NS5B for both genotypes arose predominantly at amino acid 495, where the different relative frequencies of S/L/A variants correlated with different levels of resistance. However, an additional NS5B substitution (L392I) occurred in the gt 1a NS5A/NS5B dual selection, generating a variant with low-level resistance and suggesting that alterations to NS5A, either through inhibitor interactions or via resistance substitutions, can affect emergence of resistance in NS5B, consistent with reports of NS5A-NS5B interactions (48, 55).

From the mutations that emerged only in dual selections, we can infer that cross-target influences allowed the different substitutions to emerge. Given that the nonstructural proteins targeted by these DAAs are believed to function together as a multicomponent complex, one can envision that simultaneously targeting multiple proteins may necessitate additional changes to allow formation of an active replicase complex. Others have also reported novel mutations (5) or increased prevalence of minor variants (29) in dual-DAA-selected populations. Although the overall differences between singly and dually treated populations in the sequence and resistance levels we observed were not dramatic, they alert us to the potential for unexpected outcomes during combination treatment. The results indicate that the replicon may not tolerate more-dramatic differences, given that the target proteins still need to associate into an active replication complex. Whether the mutations that emerged *in vitro* as a result of dual- and triple-DAA challenges would also be observed in patients treated with the same combinations is to be determined as clinical data are reported. The HCV replicon system should be extremely useful for analyzing samples from subjects treated with combinations of DAAs.

Interesting differences between monotherapy and dual-therapy treatment in replication fitness were also observed. For example, in both gt 1a and gt 1b, selection with ASV alone elicited the NS3 resistance substitution, which is "less costly" in terms of fitness. However, in the presence of both DCV and ASV, the more costly substitution appears to have been compensated for by the presence of additional substitutions in the second protein. For example, in gt 1b, N77S-R155Q replicates with ≤10% the efficiency of the WT replicon but, when linked with NS5A substitutions, the dually resistant variants replicated at least 3× more efficiently. Such compensatory changes could explain why cells treated with either DCV or ASV developed >100-fold resistance whereas cultures treated in parallel with both inhibitors developed only 5- to 10-fold resistance and required additional passages to develop a high level of resistance. More time may be needed to derive the correct mix of compatible substitutions whose combined fitness costs are not overwhelming. Likewise, the poor

(<10%) replication of the Q30H-K68R variant selected in gt 1a with DCV treatment suggests that an additional change outside NS5A must have been present in the selected cell line to enhance replication. The increased (30% to 70% compared to the WT level) replication of the dually selected D168V/Q30H-K68R variant compared to variants with the same changes in the individual NS3 and NS5A proteins (both at <10% of the WT level) was noteworthy. This finding suggests the presence of NS3-NS5A cross-target influences that enhance efficiency of the replication complex.

The high clearance efficiency of the triple combination probably reflects the reduced frequency of the multiple genetic events necessary to generate a viable triply resistant HCV replicon. However, variants resistant to all three inhibitors were eventually selected, and these had some of the same mutations that had been selected during dual-combination studies, as well as new ones. In NS3, Q80R was found to be linked to either R155Q or Q41R in gt 1b cultures maintained in the presence of the three DAAs but not in parallel cultures selected with monotherapy or 2-DAA combinations. These mutations, both previously identified as NS3 resistance sites, conferred low-level resistance on their own, and Q80R has been shown to have an auxiliary role in enhancing the resistance of R155 or D168 mutations (28, 49; F. McPhee et al., poster 1223, presented at the 46th EASL Congress, Berlin, Germany, 30 March to 3 April 2011). In gt 1a, D168E and M179T substitutions were 100% linked, as were R155K and M179A in a more highly resistant variant. In addition, both clones contained a Q89R substitution. This substitution on its own greatly enhanced replicon replication and may be essential to permit replication of these triple mutants during the increased pressure from selection with three inhibitors. A new combination of low-level NS5B resistance variants was also detected specifically in the gt 1a triple selection (T389I-A421V). The fact that the T389I-A421V substitutions enhanced (5- to 10-fold) replication of the Q30H-K68R NS5A variant suggests that, together, changes in these two proteins can form a replicase that is more active than one with only NS5A substitutions. It is of interest that, in gt 1a clones demonstrating resistance to all three inhibitors, high-level resistance to NS3 and high-level resistance to NS5B were not detected in the same clone. This may indicate that the fitness cost associated with highly resistant substitutions in three key proteins of the HCV replication machinery is too great to yield a genome capable of high replicative fitness.

Collectively, these results indicate that challenging HCV with multiple DAAs had a compelling suppressive effect on replication and elicited novel mutations and combinations of mutations. Since IFN- α and unrelated HCV inhibitor chemotypes retained activity against the multi-DAA-resistant populations generated in these experiments (data not shown), further complementation or follow-up to a combination DAA regimen should be feasible if required to achieve SVR. If the synergies demonstrated here for combinations of DAA inhibitors are predictive of reduced resistance and enhanced viral clearance in patients, a triple-combination regimen targeting three different proteins of HCV replication, and particularly including an NS5A inhibitor, promises to markedly improve the rates of SVR over α /RBV regimens, regimens that include a single DAA with or without RBV, and many dual-DAA combination regimens. Such combinations have the potential to quickly reduce viral load and limit opportunities for the emergence of multidrug resistance, helping to achieve SVR. Given the altered patterns of resistance observed during these

combination studies, these results provide guidance for resistance monitoring during ongoing combination trials.

ACKNOWLEDGMENTS

We acknowledge the technical contributions of Lourdes Valera and Bernadette Kienzle.

REFERENCES

1. Beaulieu PL. 2009. Recent advances in the development of NS5B polymerase inhibitors for the treatment of hepatitis C virus infection. *Expert Opin. Ther. Pat.* 19:145–164.
2. Blight KJ, Kolykhalov AA, Rice CM. 2000. Efficient initiation of HCV RNA replication in cell culture. *Science* 290:1972–1974.
3. Brown NA. 2009. Progress towards improving antiviral therapy for hepatitis C with hepatitis C virus polymerase inhibitors. Part I: nucleoside analogues. *Expert Opin. Invest. Drugs* 18:709–725.
4. Burton JR, Jr, Everson GT. 2009. HCV NS5B polymerase inhibitors. *Clin. Liver Dis.* 13:453–465.
5. Chase R, et al. 2009. A novel HCV NS3 protease mutation selected by combination treatment of the protease inhibitor boceprevir and NS5B polymerase inhibitors. *Antiviral Res.* 84:178–184.
6. Chou T. 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. *Pharmacol. Rev.* 58:621–681.
7. Dimitrova M, Imbert I, Kieny MP, Schuster C. 2003. Protein-protein interactions between hepatitis C virus nonstructural proteins. *J. Virol.* 77:5401–5414.
8. Egger D, et al. 2002. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. *J. Virol.* 76:5974–5984.
9. Failla C, Tomei L, De Francesco R. 1994. Both NS3 and NS4A are required for proteolytic processing of hepatitis C virus nonstructural proteins. *J. Virol.* 68:3753–3760.
10. Flint M, et al. 2009. Selection and characterization of hepatitis C virus replicons dually resistant to the polymerase and protease inhibitors HCV-796 and boceprevir (SCH 503034). *Antimicrob. Agents Chemother.* 53:401–411.
11. Foote BS, Spooner LM, Belliveau PP. 9 August 2011, posting date. Boceprevir: a protease inhibitor for the treatment of chronic hepatitis C. *Ann. Pharmacother.* 45:1085–1093.
12. Fridell RA, Qiu D, Wang C, Valera I, Gao M. 2010. Resistance analysis of the HCV NS5A inhibitor BMS-790052 in the *in vitro* replicon system. *Antimicrob. Agents Chemother.* 54:3641–3650.
13. Fridell RA, et al. 2011. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: *in vitro* and *in vivo* correlations. *Hepatology* 54:1924–1935.
14. Gane EJ, et al. 2010. 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* 376:1467–1475.
15. Gao M, et al. 2010. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* 465:96–100.
16. Gentile I, Carleo MA, Borgia F, Castaldo G, Borgia G. 2010. The efficacy and safety of telaprevir—a new protease inhibitor against hepatitis C virus. *Expert Opin. Invest. Drugs* 19:151–159.
17. Grakoui A, McCourt DW, Wychowski C, Feinstone SM, Rice CM. 1993. A second hepatitis C virus-encoded proteinase. *Proc. Natl. Acad. Sci. U. S. A.* 90:10583–10587.
18. Jacobson IM, et al. 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. *N. Engl. J. Med.* 364:2405–2416.
19. Kieffer TL, Kwong AD, Picchio GR. 2010. Viral resistance to specifically targeted antiviral therapies for hepatitis C (STAT-Cs). *J. Antimicrob. Chemother.* 65:202–212.
20. Kneteman NM, et al. 2009. HCV796: a selective nonstructural protein 5B polymerase inhibitor with potent anti-hepatitis C virus activity *in vitro*, in mice with chimeric human livers, and in humans infected with hepatitis C virus. *Hepatology* 49:745–752.
21. Kronenberger B, Zeuzem S. 2008. Future treatment options for HCV: double, triple, what is the optimal combination? *Best Pract. Res. Clin. Gastroenterol.* 22:1123–1136.

22. Kronenberger B, Zeuzem S. 2009. Treatment of chronic hepatitis C: anticipated impact of resistance in patients treated with protease inhibitors. *Curr. Gastroenterol. Rep.* 11:15–21.
23. Kukulj G, et al. 2005. Binding site characterization and resistance to a class of non-nucleoside inhibitors of the hepatitis C virus NS5B polymerase. *J. Biol. Chem.* 280:39260–39267.
24. Kwong AD, Kim JL, Rao G, Lipovsek D, Raybuck SA. 1999. Hepatitis C virus NS3/4A protease. *Antiviral Res.* 41:67–84.
25. Lamarre D, et al. 2003. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* 426:186–189.
26. Legrand-Abraham F, Nicot F, Izopet J. 2010. New NS5B polymerase inhibitors for hepatitis C. *Expert Opin. Invest. Drugs* 19:963–975.
27. Lemm JA, et al. 2010. Identification of hepatitis C virus NS5A inhibitors. *J. Virol.* 84:482–491.
28. Lenz O, Verhinnen et al. 2010. *In vitro* resistance profile of the hepatitis C virus NS3/4A protease inhibitor TMC435. *Antimicrob. Agents Chemother.* 54:1878–1887.
29. Le Pogam S, et al. 2006. Selection and characterization of replicon variants dually resistant to thumb- and palm-binding nonnucleoside polymerase inhibitors of the hepatitis C virus. *J. Virol.* 80:6146–6154.
30. Liu K. 2010. Development of novel antiviral therapies for hepatitis C virus. *Virol. Sin.* 25:246–266.
31. Liu K, Perni RB, Kwong AD, Lin C. 2006. VX-950, a novel hepatitis C virus (HCV) NS3-4A protease inhibitor, exhibits potent antiviral activities in HCV replicon cells. *Antimicrob. Agents Chemother.* 50:1813–1822.
32. Liverton NJ, et al. 2010. MK-7009, a potent and selective inhibitor of hepatitis C virus NS3/4A protease. *Antimicrob. Agents Chemother.* 54:305–311.
33. Lohmann V, Koch JO, Bartenschlager R. 1996. Processing pathways of the hepatitis C virus proteins. *J. Hepatol.* 24(2 Suppl):11–19.
34. Lok AS, et al. 2012. Preliminary study of two antiviral agents for hepatitis C genotype 1. *N. Engl. J. Med.* 366:216–224.
35. Love RA, Brodsky O, Hickey MJ, Wells PA, Cronin CN. 2009. Crystal structure of a novel dimeric form of NS5A domain I protein from hepatitis C virus. *J. Virol.* 83:4395–4403.
36. Lu L, et al. 2004. Mutations conferring resistance to a potent hepatitis C virus serine protease inhibitor *in vitro*. *Antimicrob. Agents Chemother.* 48:2260–2266.
37. Macdonald A, Harris M. 2004. Hepatitis C virus NS5A: tales of a promiscuous protein. *J. Gen. Virol.* 85(Pt 9):2485–2502.
38. McPhee F, et al. 2012. Resistance analysis of the hepatitis C virus NS3 protease inhibitor asunaprevir. *Antimicrob. Agents Chemother.* 56:3670–3681.
39. McPhee F, et al. 2009. The discovery and early development of the HCV NS3 protease inhibitor BMS-605339. *Global Antiviral J.* 5(Suppl 1):51.
40. Miyazaki Y, et al. 2007. The lipid droplet is an important organelle for hepatitis C virus production. *Nat. Cell Biol.* 9:1089–1097.
41. Neddermann P, Clementi A, De Francesco R. 1999. Hyperphosphorylation of the hepatitis C virus NS5A protein requires an active NS3 protease, NS4A, NS4B, and NS5A encoded on the same polyprotein. *J. Virol.* 73:9984–9991.
42. Nettles RE, et al. 2011. Multiple ascending dose study of BMS-790052, a nonstructural protein 5A replication complex inhibitor, in patients infected with hepatitis C virus genotype 1. *Hepatology* 54:1956–1965.
43. O'Boyle DR, Jr, et al. 2005. Development of a cell-based high-throughput specificity screen using a hepatitis C virus-bovine viral diarrhea virus dual replicon assay. *Antimicrob. Agents Chemother.* 49:1346–1353.
44. Ohara F, et al. 2011. Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice. *J. Hepatol.* 54:872–878.
45. Pasquinelli C, et al. 2012. Single- and multiple-ascending-dose studies of the NS3 protease inhibitor asunaprevir in subjects with or without chronic hepatitis C. *Antimicrob. Agents Chemother.* 56:1838–1844.
46. Pierra C, et al. 2006. Synthesis and pharmacokinetics of valopicitabine (NM283), an efficient prodrug of the potent anti-HCV agent 2'-C-methylcytidine. *J. Med. Chem.* 49:6614–6620.
47. Poordad F, et al. 2011. Boceprevir for untreated chronic HCV genotype 1 infection. *N. Engl. J. Med.* 364:1195–1206.
48. Qin W, et al. 2001. Mutational analysis of the structure and functions of hepatitis C virus RNA-dependent RNA polymerase. *Hepatology* 33:728–737.
49. Romano KP, Ali A, Royer WE, Schiffer CA. 2010. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. *Proc. Natl. Acad. Sci. U. S. A.* 107:20986–20991.
50. Rydberg EH, et al. 2009. Structural basis for resistance of the genotype 2b hepatitis C virus NS5B polymerase to site A non-nucleoside inhibitors. *J. Mol. Biol.* 390:1048–1059.
51. Sarrazin C, Zeuzem S. 2010. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* 138:447–462.
52. Sarrazin C, Hézode C, Zeuzem S, Pawlotsky JM. 2012. Antiviral strategies in hepatitis C virus infection. *J. Hepatol.* 56(Suppl):S88–S100.
53. Seiwert SD, et al. 2008. Preclinical characteristics of the hepatitis C virus NS3/4A protease inhibitor ITMN-191 (R7227). *Antimicrob. Agents Chemother.* 52:4432–4441.
54. Shepard CW, Finelli L, Alter MJ. 2005. Global epidemiology of hepatitis C virus infection. *Lancet Infect. Dis.* 5:558–567.
55. Shirota Y, et al. 2002. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. *J. Biol. Chem.* 277:11149–11155.
56. Soriano V, Peters MG, Zeuzem S. 2009. New therapies for hepatitis C virus infection. *Clin. Infect. Dis.* 48:313–320.
57. Susser S, et al. 2009. Characterization of resistance to the protease inhibitor boceprevir in hepatitis C virus-infected patients. *Hepatology* 50:1709–1718.
58. Tellinghuisen T, Foss KL, Treadaway J. 2008. Regulation of hepatitis C virus production via phosphorylation of the NS5A protein. *PLoS Pathog.* 4:e1000032. doi:10.1371/journal.ppat.1000032.
59. Tellinghuisen TL, Marcotrigiano J, Rice CM. 2005. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. *Nature* 435:374–379.
60. Thompson AJ, McHutchison JG. 2009. Antiviral resistance and specifically targeted therapy for HCV (STAT-C). *J. Viral Hepatol.* 16:377–387.
61. Tong X, et al. 2008. Characterization of resistance mutations against HCV ketoamide protease inhibitors. *Antiviral Res.* 77:177–185.
62. Zheng X, et al. 2011. Syntheses and initial evaluation of a series of indolofused heterocyclic inhibitors of the polymerase enzyme (NS5B) of the hepatitis C virus. *Bioorg. Med. Chem. Lett.* 21:2925–2929.