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SUPERINTENDENCIA DE INDUSTRIA Y COMERCIO



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Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____ D. _____

Re: PI.-F. HOFFMANN-LA ROCHE LTD., ROCHE PALO ALTO LLC Y PHARMASSET, INC.-Solicitud de Patente de Invención en Fase Nacional PCT para "COMPOSICIÓN FARMACEÚTICA QUE COMPRENDE LA COMBINACIÓN DE DIISOBUTIL ÉSTER DE β -D-2'-DESOXI-2'-FLUORO-2'-C-METILCITIDINA Y 14-TER-BUTOXICARBONILAMINO-4-CICLOPROPANSULFONILAMINOCARBONIL-2,15-DIOXO-3,16-DIAZA-TRICICLO[14,3,0,04,6]NONADEC-7-EN-18-IL ÉSTER DEL ÁCIDO 1S, 4R, 6S, 14S, 18R)-4-FLUORO-1,3-DIHIDRO-ISOINDOL-2-CARBOXÍLICO".-Clase A61K 31/4709.-Expediente número 11-108.893.

Yo, **ALICIA LLORED A RICAURTE**, mayor, vecina de esta ciudad, identificada con la cédula de ciudadanía número 39.690.713 de Usaquén, Representante Legal en Bogotá para todos los asuntos relacionados con la Propiedad Industrial de la sociedades **F. HOFFMANN-LA ROCHE LTD., ROCHE PALO ALTO LLC Y PHARMASSET, INC.** La primera organizada y existente de conformidad con las leyes de Suiza y domiciliada en Basilea, Suiza; y las dos últimas, organizadas y existentes de conformidad con las leyes del Estado de Delaware, Estados Unidos de América; domiciliadas en Palo Alto, Estado de California, Estados Unidos de América y Princeton, Estado de New Jersey, Estados Unidos de América respectivamente; atentamente manifiesto a usted que interpongo **RECURSO DE REPOSICIÓN** contra la Resolución Número **60350**, dictada por el señor Superintendente de Industria y Comercio el 17 de Octubre de 2013, en relación con la negación del privilegio de patente de invención para la solicitud en referencia, por una supuesta falta de **Nivel Inventivo** de dicha solicitud.

I. FUNDAMENTOS DE DERECHO

Me permito manifestar ante ese Despacho que el mencionado Recurso de Reposición encuentra fundamento en que el contenido de la Resolución Número **60350**

evidencia que durante el examen técnico realizado a la solicitud denegada se realizó una evaluación incorrecta del objeto de la invención allí reclamada, sin considerarse su real alcance e importancia.

Es así que, a pesar de los argumentos proporcionados por el solicitante en mi memorial de Septiembre 10 de 2013 (en respuesta al Oficio No. 10596), en la Resolución impugnada ese Despacho mantiene las objeciones frente al Nivel Inventivo de la invención, afirmando categóricamente que la persona versada en la materia a partir de las enseñanzas de D2 y D4 podría predecir las características de la composición que hace parte de la composición de la presente invención, concluyendo lo siguiente:

"La persona del oficio normalmente versada en la materia, al observar la combinación de danoprevir y mericitabina, tal como se revela en D2 con actividad antiviral contra hepatitis C, habría podido proponer una composición farmacéutica tal como se sugiere en D4, como una alternativa para así llegar al objeto de la reivindicación 1 de la solicitud. Por lo cual se considera obvia."

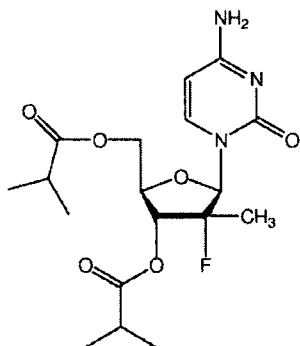
En vista de lo anterior, a continuación me permito brindar argumentos técnicos que no sólo demostrarán a ese Despacho que la presente invención sí cumple con el requisito de Nivel Inventivo exigido por el Artículo 18 de la Decisión 486 de 2000, sino que además establecerán que el análisis realizado es incorrecto, toda vez que se interpreta de manera errónea tanto la invención como el arte previo; constituyéndose por lo tanto la negación de la solicitud en una clara violación al mencionado Artículo 18 de la Decisión Andina 486 de 2000.

1. Principales características de la invención denegada

Ahora bien, con el fin de contextualizar los motivos por los cuales ese Despacho realiza una incorrecta valoración y evaluación del arte previo, me permito señalar a continuación las principales características y ventajas técnicas de la materia revelada en la solicitud de patente negada.

La presente solicitud se relaciona con una novedosa composición caracterizada porque comprende:

un primer compuesto, o una de sus sales farmacéuticamente aceptables, donde el primer compuesto es

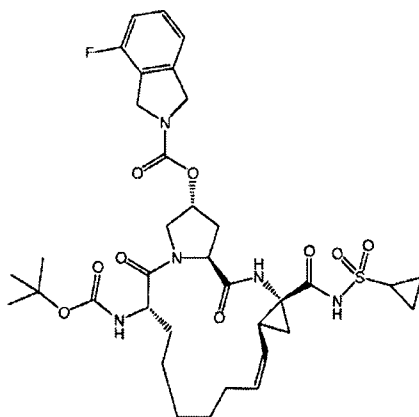


Compuesto 1a

el cual se encuentra en una cantidad de 9000 mg a 50 mg;

un segundo compuesto, o una de sus sales

farmacéuticamente aceptables, donde el segundo compuesto es



Compuesto 2

el cual se encuentra en una cantidad de 2000 mg a 2 mg; y un excipiente, diluyente o portador farmacéuticamente aceptable.

Dichos compuestos actúan como un inhibidor de nucleósido de polimerasa y como un inhibidor de proteasa macrocíclica respectivamente útiles en el tratamiento de la hepatitis C.

Es así, como la invención reclamada en la presente solicitud se encuentra dirigida a proteger una novedosa composición que comprende la combinación de dos agentes activos específicos como son el compuesto 1a y el compuesto 2, los cuales adicionalmente son efectivos en el tratamiento de fibrosis hepática e insuficiencia hepática, como se demuestra a través de los resultados clínicos descritos en la memoria descriptiva en las páginas 54 a 69 de la presente solicitud.

En este sentido me permito resaltar que estas propiedades mejoradas de la combinación de la invención fueron comprobadas por el solicitante, tal como lo evidencia la descripción de la solicitud y los argumentos proporcionados en respuesta al primer requerimiento técnico, y dichas propiedades no pueden de ninguna manera ser

evidentes a partir de las enseñanzas del arte previo, lo que será demostrado a continuación.

2. Consideraciones técnicas frente a las enseñanzas del arte previo.

Habiendo ya contextualizado el objeto e impacto que tiene la solicitud denegada, me permito reiterar que el objeto de dicha solicitud es a todas luces inventivo con respecto a las enseñanzas del arte previo citado en la Resolución Número **60350**, esto es, los documentos **"Recent progress in the development of selected hepatitis C virus NS3.4A protease and NS5B polymerase inhibitors" (D2)** y **WO 2005 003147 (D4)**.

Con el fin de demostrar este hecho, a continuación me permito exponer las principales razones que harían que la persona versada en la materia no pudiera de ninguna forma obtener la materia reclamada en la solicitud de negada partiendo de los citados documentos D2 y D4, evidenciando así la incorrecta interpretación que realiza ese Despacho del arte previo, interpretación que lo lleva a concluir de forma errónea con respecto a la patentabilidad de la solicitud.

➤ Con respecto al documento D2, en la Resolución número **60350** se afirma que:

"La diferencia entre la invención, definida en la reivindicación 1 y la combinación de D2, consiste en que no se mencionan composiciones juntos a excipientes, diluyentes o portadores que contengan estos principios activos"

Con respecto a lo anterior considero de gran importancia aclarar ante ese Despacho lo siguiente:

(a) En efecto, es importante resaltar que las composiciones reclamadas en la presente solicitud no difieren únicamente del documento D2, en la presencia de un excipiente, diluyente o vehículo, como se alega en la Resolución **60350**, sino también en que, comprende tanto el compuesto 1a en combinación con el compuesto específico 2 en una única composición, que no son conocidos en el documento D2.

Es así, como el documento D2 corresponde artículo de revisión publicado en el año 2008, en donde analiza los últimos avances en el desarrollo de inhibidores de proteasa del virus de hepatitis (VHC) NS3/4A e inhibidores de polimerasa NS5B, tal y como se evidencia en el título del documento. Sin embargo, el documento D2 no se relaciona con un tratamiento terapéutico que emplee un inhibidor de proteasa NS3/4A en combinación con un inhibidor de polimerasa NS5B, así como tampoco con una

composición que contenga los anteriores agentes inhibidores en combinación, ni mucho menos que la composición que comprenda el compuesto específico, inhibidor de polimerasa R7128 (Compuesto 1a) en combinación específicamente con el inhibidor de proteasas ITMN-191 (Compuesto 2), como se reclamada en las actuales reivindicaciones.

En conexión con lo anterior, es absolutamente claro que el objetivo técnico del documento D2, no es abordar nuevas composiciones en combinación de agentes terapéuticos útiles en el tratamiento de la infección por el virus de VHC, sino pretende comparar y correlacionar datos clínicos y preclínicos para diversos agentes inhibidores de polimerasa e inhibidores de proteasa, este argumento se fortalece con las afirmaciones encontradas a lo largo del documento D2 que revela la existencia de múltiples publicaciones dirigidas al estudio de la interacción de una célula y el virus VHC actualmente desarrollados. Así mismo, se han enfocado en compuestos que presentan estudios clínicos avanzados, como inhibidores de polimerasa y proteasa del virus de VHC. Es así, como el objetivo de esta revisión es comparar la potencia anti-viral en clínica, con datos pre-clínicos de los inhibidores de proteasa de VHC e inhibidores de polimerasa. La Tabla 1 resume los datos de los compuestos antivirales, los cuales corresponden a múltiples ensayos clínicos comparativos y no representan comparaciones específicas entre compuestos (Ver pág. 254)

De esta forma, dentro de los compuestos inhibidores de proteasa de VHC, se encuentra el compuesto ITMN-19 y dentro de los inhibidores de polimerasa incluyen en compuesto R7128, pero estos inhibidores de polimerasa y proteasa fueron evaluados tanto en monoterapia como en la combinación con interferón pegilado (Peg-IFN) o interferón pegilado mas ribavirina (Peg-IFN/RBV), lo cual resulta evidente a partir de los regímenes de tratamiento y dosis utilizadas en los ensayos clínicos de la Tabla 1.

De esta manera, es importante resaltar que documento D2 no enseña ni sugiere una combinación de inhibidores de proteasa de VHC con un inhibidor de polimerasa, y mucho menos la combinación específica del compuesto R7128 (compuesto 1a) con ITMN-19 (compuesto 2).

En conexión con lo anterior, el documento D2 enseña la comparación de potencia antiviral *in vivo* e *in vitro* de inhibidores de proteasa de VHC con un inhibidor de polimerasa, de la Figura 3. (Pág. 527) en donde los datos reportados de actividad corresponden a cada uno de los compuestos inhibidores empleados en monoterapia y NO para una combinación particular de un inhibidor de proteasa de VHC con un inhibidor de polimerasa. Esto es claramente evidente a la luz de la Figura 3. (a), donde una de las columnas hace referencia a la monoterapia bajo el encabezado "*in vivo* (monoterapia)". De igual forma, cuando se refiere al análisis de la figura 3., explica que

los datos correspondientes se derivan de los tratamientos en monoterapia. (Pág. 527; Columna Izquierda; Línea 8 a 13).

En vista de lo anterior, el documento D2 una vez más demuestra que no es sugerida ni divulgada una composición que comprenda el compuesto 1a (R7128) en combinación con el compuesto 2 (ITMN-191), tal y como se reclama en la presente solicitud. De hecho, la única a una combinación, adicional a la combinación mencionada con un Peg-IFN/RBV, se encuentra en el último párrafo del documento D2, bajo el título "*Necesidades médicas no satisfechas y los retos futuros*", en donde se refiere a la futura introducción de combinaciones de agentes antivirales que presenten potencia aditiva y no presenten resistencia al virus, de esta forma el documento D2 afirma que "*tenemos que ser innovadores y proactivos probar estas combinaciones una vez se disponga de datos suficientes de los agentes individuales*". (Pág. 528; Columna derecha), lo que se entiende como un desarrollo a futuro y que no hace parte de las enseñanzas presentes del documento D2.

Aun así, cuando se realiza la lectura atenta del documento D2, se evidencia que ni siquiera cuando se refiere a desarrollos futuros, los autores mencionan una composición que comprenda específicamente a un inhibidor de proteasa en combinación con un inhibidor de polimerasa, ni mucho menos la composición tal y como se reclama en la presente invención que comprende el compuesto R7128 (compuesto 1a), en combinación con el compuesto ITMN-191 (compuesto 2). Muy por el contrario, a lo largo del documento D2, se reconoce que la combinación de agentes activos contra hepatitis C es beneficiosa, y que corresponde a un desarrollo esperado a largo plazo, siendo necesaria la innovación orientada a combinaciones beneficiosas.

Contrario a las enseñanzas mencionadas, los inventores han encontrado de manera sorprendente las ventajas de la eficacia terapéutica de la composición actualmente reclamada, asociada con el tratamiento o mejora de enfermedades que incluyen infecciones por el virus de la hepatitis C, el cual ha sido demostrado clínicamente con los datos proporcionados en la presente solicitud (págs. 54 – 55, y Tabla 2. pág. 64), los cuales de ninguna manera pueden ser esperados de manera obvia a partir de las enseñanzas del documento D2.

Por otra parte, la eficacia terapéutica mejorada de la composición de la presente solicitud, se confirma con las enseñanzas del documento Anexo I. el cual divulga que la administración combinada de los activos mericitabina (compuesto 1a) con danoprevir (compuesto 2), en pacientes con infección crónica por el VHC, en un estudio doble ciego controlado con placebo, disminuye la carga viral del virus VHC, y además previene o reduce la aparición de la resistencia a danoprevir.

En conexión con lo anterior y a la luz de las enseñanzas del documento D2, es evidente que la persona normalmente versada en la materia, no habría derivado de manera obvia la composición tal y como se reclama en la presente solicitud, ni mucho menos obtener las sorprendentes ventajas terapéuticas logradas con la composición, máxime cuando el documento D2 no sugiere ni divulga la combinación específica de los compuestos 1a y el compuesto 2 en una composición.

Finalmente, además de que el documento D2 no afecta el nivel inventivo de la solicitud, consideramos oportuno aclarar, que en ningún momento a lo largo del trámite administrativo ante ese Despacho, el documento D2 fue citado como un documento que afectara el nivel inventivo de la composición reivindicada, toda vez que dicho documento había sido mencionado ÚNICAMENTE para afectar la Novedad, la cual ha sido superada en virtud del capítulo reivindicatorio que se presentó en su momento.

En este sentido, solo hasta la Resolución de negación, aquí impugnada, por primera vez el examinador hace mención a que D2 afecta el nivel inventivo de la invención, lo cual no es un argumento nuevo, pues durante el trámite, solo mencionó dicho documento por novedad, quedando así aún más claro que D2 no afecta el nivel inventivo de la solicitud, y mucho menos en combinación con D4.

➤ Con respecto al documento D4, en la Resolución número **60350** se afirma que:

"la diferencia entre la invención, definida en la reivindicación 1 y las composiciones de D4 consiste en que no menciona como agente en las composiciones el compuesto danoprevir"

Con respecto a esta afirmación es importante aclarar que el documento D4 se refiere a compuestos estructuralmente relacionados con el compuesto 1 de la presente solicitud, pero no divulga ni sugiere específicamente el compuesto 1a, ni mucho menos menciona nada referente a la combinación de los compuestos específicos 1a y 2, tal y como se reclama en la presente invención.

De esta manera y de acuerdo con lo mencionado anteriormente es posible afirmar que a partir de las enseñanzas de los documentos citados en la Resolución número **60350**, para la persona normalmente versada en la materia no es posible derivar de manera obvia la composición en combinación que comprenda todas y a cada una de las características técnicas de la composición reclamada en la presente solicitud, así como tampoco es obvio predecir que a partir de la combinación de compuesto 1a con el compuesto 2, se obtendrían resultados clínicos efectivos asociados con el tratamiento o mejoría de condiciones como las de la infección por el virus VHC, máxime cuando los

documentos D2 ni D4 sugieren o divulgan la combinación de compuestos inhibidores de proteasa de VHC con un compuesto inhibidor de polimerasa, tal y como se reclaman en presente solicitud.

3. Requisitos exigidos por el Artículo 18, la evaluación del Nivel Inventivo de una Solicitud de patente.

Dentro del contexto de los argumentos discutidos anteriormente, me permito señalar que el Artículo 18 de la Decisión Andina 486 de 2000 exige de forma manifiesta que la invención no debe resultar obvia ni se debe poder derivar de forma evidente de lo existente en el arte previo, y por lo tanto es en este sentido que el examinador debe encaminar su análisis para sopesar si una solicitud es o no merecedora del beneficio de patente.

Así mismo, me permito llamar la atención a su Despacho a que el Tribunal de Justicia de la Comunidad Andina se ha pronunciado con respecto a las definiciones de "obvio" y "evidente" en la evaluación del Nivel Inventivo de una solicitud de patente¹:

"Resulta pertinente, a los efectos del artículo 4 de la Decisión 344, definir qué se entiende por "obvio" y por "evidente" a fin de determinar qué se debe entender por Nivel Inventivo.

Lo obvio es aquello que salta a la vista de manera clara y directa; lo "que se encuentra o pone delante de los ojos, muy claro o que no tiene dificultad". Mientras que lo evidente consiste en la "certeza clara y manifiesta de la que no se puede dudar; cierto, claro, patente y sin la menor duda". De forma que algo que resulte obvio no es necesariamente evidente; empero lo que es evidente, es también obvio.

En este orden de ideas, se puede concluir que una invención goza de Nivel Inventivo cuando a los ojos de un experto medio en el asunto de que se trate, se necesita algo más que la simple aplicación de los conocimientos técnicos en la materia para llegar a ella, es decir, que de conformidad con el estado de la técnica el invento no sea consecuencia clara y directa de dicho estado."

Con respecto a este señalamiento me permito resaltar que resulta indiscutible que para la legislación Andina, para que exista afectación del requisito de Nivel Inventivo de una solicitud de patente, es necesario que la invención allí revelada sea derivable de forma clara y directa del arte previo, y que las enseñanzas de este arte previo sin lugar a dudas conduzcan a dicha invención.

¹ Tribunal de Justicia de la Comunidad Andina, PROCESO 192-IP-2005

Consecuentemente, para el caso que nos compete dado que a partir de las enseñanzas del arte previo citado no "salta a la vista de manera clara y directa" la composición que comprenda las características técnicas de aquella reclamada por la presente invención, y mucho menos a partir de las enseñanzas de estos documentos se tiene "certeza clara y manifiesta" de que ésta combinación sería efectiva particularmente en el tratamiento o mejoría de la infección del virus por el VHC, es evidente que el arte previo no puede afectar el Nivel Inventivo de la solicitud en estudio.

En conexión con lo anterior me permito muy respetuosamente señalar que en su análisis de Nivel Inventivo de la solicitud en estudio ese Despacho incurre en lo que dentro del ámbito de las patentes se conoce como "hindsight", o análisis "Ex post facto", toda vez que conociendo de antemano la invención selecciona partes específicas de las enseñanzas contenidas dentro de los documentos citados, agrupándolas de una manera particular y modificándolas de forma que permitan obtener la composición reclamada por la presente solicitud, pasos que de ningún modo podrían ser evidentes para la persona versada en la materia en el momento en el cual se desarrolló la invención.

En este sentido vale la pena resaltar que al tener conocimiento de la invención es de esperarse que dicha selección, agrupación y modificaciones particulares de las enseñanzas de D2 y D4 parezcan obvias, toda vez que ya se sabe de antemano que efectivamente estos elementos en combinación resuelven el problema planteado; sin embargo, esta información no era conocida por el inventor en el momento de desarrollar la invención, por lo que no podría tener certeza de la utilidad de realizar estos pasos, máxime cuando D2 y D4 no revelan ni sugieren combinaciones de compuestos específicamente los reclamados en la solicitud denegada.

En vista de lo anterior, dado que la única motivación que tendría la persona versada en la materia de reunir y modificar estas características particulares reveladas en el arte previo sería la misma invención como ha sido revelada, es evidente el Nivel Inventivo que conlleva dicha solicitud.

II. CONCLUSIONES

Con base en los argumentos técnicos mostrados previamente, me permito manifestar ante ese Despacho que la materia revelada en la solicitud de patente en cuestión, presenta un inequívoco carácter inventivo frente a las enseñanzas del arte previo citado en la Resolución Número **60350** aquí impugnada, cumpliendo así a cabalidad las disposiciones establecidas en el Artículo 18 de la Decisión Andina 486 de 2000, y por lo tanto demostrando ser merecedora del privilegio de patente requerido.

En efecto, los argumentos proporcionados demostraron que ese Despacho interpreta de forma errónea las revelaciones de los documentos citados, comparando de manera independiente un inhibidor de proteasa de VHC con un inhibidor de polimerasa y no en una combinación de los mismos.

De otra parte, se demostró que aún si se tomaran como base las revelaciones de D2 y D4 para obtener la composición de la invención, dichos documentos no sugieren de manera específica las características la invención tal y como es reclamada por la solicitud denegada.

Como consecuencia de todo lo anterior, me permito resaltar que dado que la solicitud denegada sí se ajusta completamente a los requerimientos establecidos por la Decisión Andina 486, al negar el privilegio de patente ese Despacho claramente contraviene los Artículos 14 y 18 de dicha Decisión.

III. SOLICITUD

En vista de todo lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de las argumentaciones presentadas y revocar la Resolución Número **60350** impugnada, y en consecuencia conceder el privilegio de patente solicitado.

IV. PODER

En este punto me permito mencionar ante ese Despacho que el documento que acredita mi calidad de Representante Legal en Bogotá para asuntos relativos a la Propiedad Industrial de la sociedad peticionaria fue presentado junto con mi memorial de 20 de Diciembre de 2011.

V. ANEXOS

Anexo me permito presentar el siguiente artículo

- Anexo I. S. Le Pogam, J. M. Yan, M. Chhabra, M. Ilnicka, H. Kang. A Kosaka, S. Ali, D. J. Chin, N. S. Shulman, P. Smith, K. Klumpp and I. Nájera. Characterization of Hepatitis C Virus (HCV) Quasispecies Dynamics upon Short-Term Dual Therapy with the HCV NS5B Nucleoside Polymerase Inhibitor Mericitabine and the NS3/4 Protease Inhibitor Danoprevir. Antimicrobial Agents and Chemotherapy. P 5494 – 5502. November 2012. Volume 56. Number 11.

VI. DOMICILIO

Mi dirección es Calle 72 No. 5-83, Piso 6º, Bogotá D.C.

Del Señor Superintendente,



ALICIA LLOREDA RICAURTE

T.P. 53.215

Antimicrobial Agents
and Chemotherapy

**Characterization of Hepatitis C Virus (HCV)
Quasispecies Dynamics upon Short-Term
Dual Therapy with the HCV NS5B
Nucleoside Polymerase Inhibitor
Mericitabine and the NS3/4 Protease
Inhibitor Danoprevir**

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Characterization of Hepatitis C Virus (HCV) Quasispecies Dynamics upon Short-Term Dual Therapy with the HCV NS5B Nucleoside Polymerase Inhibitor Mericitabine and the NS3/4 Protease Inhibitor Danoprevir

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In the INFORM-1 study, 73 patients with chronic hepatitis C virus infection received mericitabine plus danoprevir for up to 13 days. Seventy-two patients experienced a continuous decline in HCV RNA levels during treatment, and of these patients, 14 had viral loads that remained >1,000 IU/ml by day 13 and 1 met the definition for viral breakthrough. In-depth NS5B and NS3/4A population and clonal sequencing studies and mericitabine and danoprevir drug susceptibility testing were performed to assess the variability and quasispecies dynamics before and upon monotherapy or dual therapy. Sequence analysis of the viral quasispecies indicated that the mericitabine resistance mutation S282T was not present at baseline, nor was it selected (even at a low level) during treatment. Protease inhibitor resistance mutations, either as predominant or as minority species, were detected in 18 patients at baseline. No enrichment of minority protease inhibitor-resistant variants present at baseline was observed during treatment; viral population samples were fully susceptible to mericitabine and/or danoprevir, despite the presence within their quasispecies of minority variants confirmed to have reduced susceptibility to danoprevir or other protease inhibitors. It was also observed that certain NS3 amino acid substitutions affected protease inhibitor drug susceptibility in a compound-specific manner and varied with the genetic context. In summary, the slower kinetics of viral load decline observed in some patients was not due to the selection of danoprevir or mericitabine resistance during treatment. Over 2 weeks' therapy, mericitabine suppressed the selection of danoprevir resistance, results that could differ upon longer treatment periods.

Chronic infection with hepatitis C virus (HCV) is a leading cause of liver disease worldwide (10). With the approval of the first HCV NS3/4A protease inhibitors (PIs; boceprevir and telaprevir), which are used in combination with pegylated interferon (peginterferon) and ribavirin, cure rates for treatment-naïve HCV genotype 1-infected patients have improved to ~70% (11, 15, 30). However, given the high genetic variability and population turnover of HCV, these PIs have a low barrier to the selection of PI-resistant variants, even when administered in combination with peginterferon and ribavirin (13, 28, 38). Danoprevir (RG7227) is a macrocyclic HCV protease inhibitor (3, 4). Among treatment-naïve and treatment-experienced patients receiving danoprevir monotherapy for 14 days, the incidence of viral breakthrough was 27%, and the incidence of partial response was 35%. Viral breakthrough in these patients was associated with the emergence of the NS3 protease variant R155K (22).

Mericitabine (RG7128) is a nucleoside inhibitor of the HCV NS5B polymerase that acts as an RNA chain terminator and prevents elongation of RNA transcripts during replication (12, 24, 29, 37). Resistance to mericitabine *in vitro* is conferred by the NS5B substitution S282T (1), but no resistance-related viral breakthrough has been observed in patients treated for 2 weeks with mericitabine alone as monotherapy or for up to 24 weeks with mericitabine in combination with peginterferon-ribavirin (12, 19, 21, 29). In the phase IIb INFORM-SVR study, which investigated 12 or 24 weeks of response-guided treatment with mericitabine and ritonavir-boosted danoprevir with and without ribavirin in treatment-naïve HCV genotype 1-infected patients, the S282T variant was described in one patient (6).

Viral kinetic models predict that every possible single, double, and triple mutant may exist in an HCV-infected patient before treatment and that, theoretically, resistant variants (if fit) can be rapidly selected during treatment (33). The selection and enrichment of a resistant variant depend on its baseline prevalence and fitness (20, 28). The emergence of resistance is a particular problem among patients who have not responded to prior treatment with peginterferon-ribavirin. Combining a compound with a high barrier to resistance, such as mericitabine with a PI, may delay or prevent the emergence of resistance to the PI, even in the absence of interferon.

The INFORM-1 study assessed the safety and efficacy of up to 13 days of treatment with mericitabine and danoprevir and demonstrated consistent reductions in HCV RNA (7). In this paper, we report the results of detailed resistance studies that were performed to determine the baseline prevalence of HCV variants with

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resistance to mericitabine, danoprevir, or other PIs (including sequence analyses of 2,937 NS3 clones from 34 samples and 1,910 NS5B clones from 21 samples). We also studied the dynamic evolution of minority variants and their impact on drug susceptibility to determine whether short-term treatment (13 days) with the combination of danoprevir and a compound with a high barrier to resistance, mericitabine, could prevent the emergence of danoprevir-resistant variants that typically emerge rapidly when danoprevir is administered alone as monotherapy.

MATERIALS AND METHODS

Patients and study design. INFORM-1 was a phase I, randomized, double-blind, placebo-controlled, dose-escalation trial (NCT00801255) in which 87 adult patients with HCV genotype 1 infection were randomized to receive up to 13 days of oral combination therapy with danoprevir and mericitabine or placebo. Patients in cohort A were randomized to receive 3 days of monotherapy with either low-dose mericitabine at 500 mg every 12 h (q12h) (cohort A1) or low-dose danoprevir at 100 mg every 8 h (q8h) (cohort A2). Both subgroups then received the combination of mericitabine plus danoprevir for an additional 4 days. In all other cohorts (cohorts B to G), patients received concurrent treatment with mericitabine at a dosage of either 500 or 1,000 mg q12h and danoprevir at a dosage of 100 or 200 mg q8h or 600 or 900 mg q12h for 13 days. The trial was conducted in accordance with the Declaration of Helsinki. The protocol was approved by regulatory authorities and by the local research ethics committees at each study site. Written informed consent was obtained from all patients before they underwent any study-related procedures. A comprehensive description of the patient selection criteria, study design, and the primary results are published elsewhere (7).

Sample selection for viral resistance monitoring studies. Baseline samples from all patients and samples from patients experiencing viral breakthrough, defined as an increase in HCV RNA level of at least 0.5 log₁₀ IU/ml above the nadir, and from patients with an end-of-monotherapy (cohort A) or end-of-treatment (all other cohorts) HCV RNA level of at least 1,000 IU/ml, were analyzed for viral resistance.

Plasmid constructions. The NS5B shuttle replicons (H77 and Con1 strains) contain restriction sites flanking the start and the end of the NS5B gene (19). The NS3 shuttle replicon based on Con1 contains restriction sites flanking the start and the end of the NS3 protease gene.

HCV RNA extraction, target gene amplification, and cloning of clinical isolates into shuttle replicon vectors for phenotypic characterization and clonal analysis. HCV RNA extraction, reverse transcription, NS5B amplification in duplicate for each isolate, and cloning were carried out as previously described (19). Amplification of NS3/4A and/or NS3 protease sequences was carried out similarly to the NS5B amplification procedure. All NS3 protease genes (genotype 1a and 1b) were cloned into the genotype 1b Con1 shuttle replicon. Briefly, to maintain the quasispecies diversity, 96 individual colonies were pooled for each clinical isolate for drug susceptibility characterization (NS5B and NS3 protease genes). Clonal analysis was performed on each of the 96 individual colonies, and the total number of successfully sequenced clones per isolate varied from 76 to 94 (NS5B and NS3 protease).

Population and clonal sequencing of target gene from clinical isolates. Population and clonal sequencing spanning the NS5B and/or NS3 protease-coding regions was performed using primers covering both DNA strands. Sequencing reactions were performed using an ABI 3730xl DNA analyzer, and chromatograms were analyzed using Sequencher and VNTI software (19).

Compounds. β-D-2'-Deoxy-2'-fluoro-2'-C-methyl cytidine (parent compound of mericitabine) was obtained from Pharmasset, Inc. (Princeton, NJ). Danoprevir was obtained from Intermune, Inc. (Brisbane, CA). Telaprevir was obtained from Acme Bioscience, Inc. (Menlo Park, CA).

Determinations of 50% effective concentrations (EC₅₀s). Four million cured Huh7 cells were transfected with 10 μg of *in vitro*-transcribed

replicon RNA from genotype 1b or genotype 1a reference strains or from clinical isolate-derived transient replicons as previously described (19).

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study have been deposited in GenBank with accession numbers JX437195 to JX437368.

RESULTS

HCV load kinetics were assessed throughout the trial. Among patients in cohort A, initial slower declines in viral load were observed in patients who received low-dose mericitabine lead-in monotherapy in cohort A1 than in patients who received low-dose danoprevir lead-in monotherapy in cohort A2. After the addition of the second drug at day 4, HCV RNA levels declined similarly in both subgroups and were generally similar at the end of dosing (day 7). The median decrease in serum HCV RNA level at the end of treatment was 3.4 log₁₀ IU/ml in cohort A1 and 3.1 log₁₀ IU/ml in cohort A2.

Among the patients in cohorts B to G who received 13 days of mericitabine-danoprevir combination therapy, the median decrease in serum HCV RNA at the end of treatment ranged from 3.7 to 5.2 log₁₀ IU/ml, with no significant differences between treatment-naïve and treatment-experienced patients (7).

Of the 73 patients who received active drug in all cohorts, 72 (98.6%) experienced a continuous decline in HCV RNA levels during treatment. Only one patient, in cohort C, who received treatment with mericitabine at 500 mg q12h and danoprevir at 200 mg q8h, met the definition for virologic breakthrough. HCV RNA levels in this individual decreased by 4.5 log₁₀ IU/ml and then increased by 1.45 log₁₀ IU/ml between the nadir and the end of dosing (day 13). Virologic breakthrough in this individual was not associated with resistance to mericitabine or danoprevir (7).

Fourteen patients had HCV RNA levels that remained above 1,000 IU/ml at the end of treatment (10 in cohort A, 4 in cohorts B to G). We wanted to determine whether the slower decline in HCV RNA in these patients was due to treatment with low drug doses, the presence of drug-resistant variants at baseline, and/or the selection of resistant variants during treatment.

Inhibition of PI-resistant virus present at baseline after short-term combination therapy. No mericitabine resistance mutation was detected in any patient by population sequencing of baseline samples. However, a number of mutations previously described to affect susceptibility to PIs were detected in 15 of 73 patients who received mericitabine plus danoprevir and 3 of 14 patients who received placebo. These included Q80K (in 8% of all patients), T54S (7%), V55A (2%), V36M (1%), Q80R (1%), R155K (1%), and D168E (1%) (3, 17, 18, 25, 26, 31, 36, 41).

Despite the presence of preexisting PI resistance mutations, HCV RNA decreased continuously in the 15 patients who received mericitabine plus danoprevir treatment and was <1,000 IU/ml in 12 of 15 patients at the end of treatment. In one patient (patient 19; Fig. 1), the danoprevir resistance mutation D168E was present in 91% of clones at baseline and was correlated with a 37-fold reduction in danoprevir susceptibility (at the population level) (7). Baseline samples (at the population level) from this patient were fully susceptible to mericitabine (0.4-fold change versus control).

No on-treatment selection of mericitabine or danoprevir resistance after 3 days monotherapy or up to 13 days dual therapy. Ten of the 17 patients in cohort A (3 who received the mericitabine lead-in in cohort A1 and 7 who received the danoprevir lead-in in

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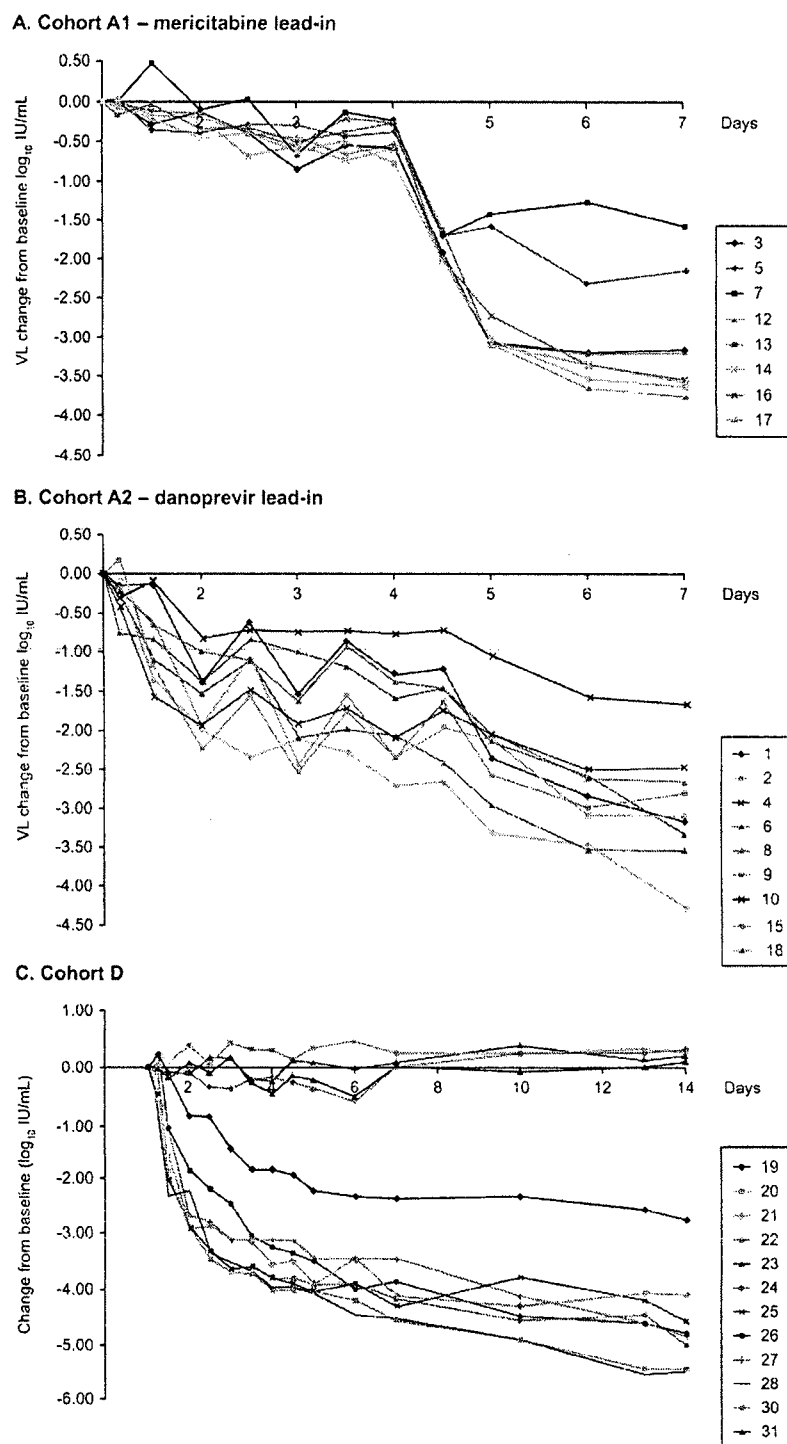


FIG 1 (A) Viral load (VL) kinetics of patients (boxed numbers) in cohort A who received mericitabine alone on days 1 to 3 and then mericitabine plus danoprevir on days 4 to 7; (B) viral load kinetics of patients in cohort A who received danoprevir alone on days 1 to 3 and then mericitabine plus danoprevir on days 4 to 7; (C) viral load kinetics of patients in cohort D. Includes a patient with a D168E resistance mutation (patient 19) who received mericitabine and danoprevir for 13 days. Other patients from the same cohort are presented as a comparison (patients 20, 23, 24, and 31 received placebo).

cohort A2) had HCV RNA levels of $>1,000$ IU/ml at the end of treatment (day 7), thus allowing PCR amplification and further analyses. Nine of these 10 patients were infected with genotype 1a.

Sequencing and phenotypic analyses of the NS3/4A and NS5B regions were performed at baseline and at treatment days 4 and 7. No mericitabine or danoprevir resistance mutations were de-

tected by population sequencing at the end of monotherapy (day 4) or at the end of dual therapy (day 7) in any of these patients (Table 1), which is in contrast to results in patients treated for 14 days with danoprevir monotherapy, among whom mutant variants were detected in approximately 50% of patients (22). Samples from all 10 patients were fully susceptible to mericitabine and

TABLE 1 Population sequence analysis of samples obtained at day 4 and 7 of treatment from 10 patients in cohort A^a

Population and patient (HCV genotype)	Treatment regimen	Day	Amino acid change(s) from baseline (day 1) sample	
			NS5B	NS3 protease
Viral load > 1,000 IU/ml on day 7				
1 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	4	K77K/R, K100K/R, H374H/Q, M423I/M, C/Y451C, K/R523K, I/M539I	Q9P/Q
		7	NA	Q9P/Q, N174N/S
2 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	4	N/S81N, C/S110C, F/Y162F, T377A, R510K/R, A/P540A	I711I/M, A/M/T/V147A, I/V170I
		7	C/S110C, Q124Q/R, F/Y162F	A/T40T, A/M/T/V147A, I/V170I
3 (1a)	Mericitabine days 1–7 + danoprevir days 4–7	4	I184I/V, F572F/I	None
4 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	7	R48R/G, K/R401R, A/T580A	None
		4	NA ^b	None
5 (1a)	Mericitabine days 1–7 + danoprevir days 4–7	7	NA	None
		4	S/T5S, S62N/S, K/R254K, S/T473T, K/R510K	None
6 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	7	S/T5S, V111I/V, M187M/V, A327A/V, S/T473T, K/R510K, K517K/R	None
		4	V71A/V, R98K/R, I520I/V, K531K/R	None
7 (1a)	Mericitabine days 1–7 + danoprevir days 4–7	7	D/G/N/S62N/S, S431G/S	I/V18V
		4	None	N174N/S
8 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	7	A185A/V, K510K/R	None
		4	S180R/S, Y276F, A/T580A	I/V114I
9 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	7	Q124Q/R, I/L462L, T520I/T, G558E/G, A/T580A	A87A/T, I/V114I
		4	N/S117S, D/E/K/N124N, Q206R, I/V405I, D444G	None ^c
10 (1b)	Danoprevir days 1–7 + mericitabine days 4–7	7	V116V/L, D/E/K/N124N, S326S/G, A334A/T, I/V405I	NA
		4	None	None
		7	None	None
Viral load < 1,000 IU/ml on day 7 (and >1,000 IU/ml on day 4)				
12 (1a)	Mericitabine days 1–7 + danoprevir days 4–7	4	S543G/S	NA
13 (1a)	Mericitabine days 1–7 + danoprevir days 4–7	4	A112A/S, N/S120N, A/G198G, F526F/L	NA
14 (1b)	Mericitabine days 1–7 + danoprevir days 4–7	4	None	V48V/I
15 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	4	NA	None
16 (1a)	Mericitabine days 1–7 + danoprevir days 4–7	4	I/V262I, F/I/S/T267T	NA
17 (1b)	Mericitabine days 1–7 + danoprevir days 4–7	4	None	None
18 (1a)	Danoprevir days 1–7 + mericitabine days 4–7	4	E/K69E, K/M72K, N130S, G549S	T95T/A

^a The patients included those with end-of-treatment serum HCV RNA levels of $\geq 1,000$ IU/ml or with HCV RNA levels of $\geq 1,000$ IU/ml at the end of monotherapy.
^b NA, sequence not available.
^c Telaprevir resistance mutation V36M was present at baseline and day 4.

danoprevir at baseline, with no change in susceptibility (at the population level) in on-treatment samples, which suggests that the amino acid substitutions observed in some samples did not alter the susceptibility to either compound (Fig. 2A and B). No

danoprevir or mericitabine resistance mutations were observed at baseline or at the end of monotherapy in any of the seven patients (five from cohort A1 and two from cohort A2) whose viral loads were >1,000 IU/ml by day 4 but <1,000 IU/ml by day 7 (Table 1).

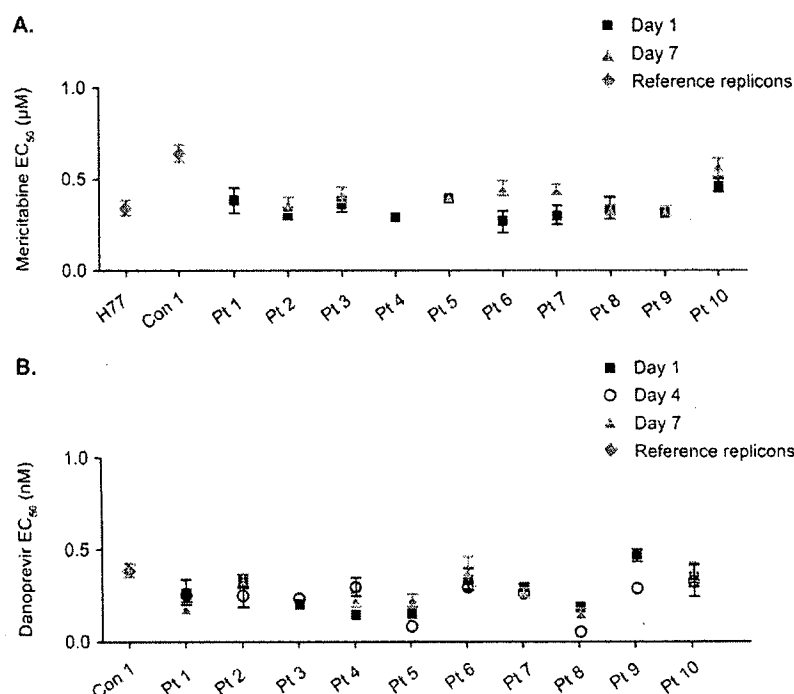


FIG 2 (A) Phenotypic characterization of susceptibility to mericitabine in samples obtained at baseline and during treatment from 10 patients in cohort A with an end-of-treatment serum HCV RNA level of $\geq 1,000$ IU/ml; (B) phenotypic characterization of susceptibility to danoprevir in samples obtained at baseline and during treatment from 10 patients in cohort A with an end-of-treatment serum HCV RNA level of $\geq 1,000$ IU/ml.

Four patients (three infected with genotype 1a and one infected with genotype 1b) in cohorts B to G who were treated for 13 days had HCV RNA levels of $>1,000$ IU/ml at the end of treatment. These included patient 40, in whom HCV RNA levels decreased by $2.9 \log_{10}$ IU/ml from baseline to 14,400 IU/ml at the end of treatment, and patients 46, 51, and 59, who experienced 2.5-, 3.5-, and $3.0 \log_{10}$ -IU/ml reductions in viral load to 2,000, 1,410, and 1,290 IU/ml, respectively, by the end of treatment (Table 1). Population and clonal sequencing at baseline did not detect any known resistance mutation in NS5B or NS3/4A. Amplification of day 14 samples from patients 46, 51, and 59 was not successful. Baseline and end-of-treatment samples from patient 40 were susceptible to both mericitabine (EC_{50} s, $0.44 \pm 0.04 \mu M$ and $0.32 \pm 0.02 \mu M$, respectively) and danoprevir (EC_{50} s, 0.37 ± 0.06 nM and 0.83 ± 0.08 nM, respectively).

Quasispecies composition and dynamics after 3 days of monotherapy followed by 4 days of dual therapy. Clonal analysis (76 to 94 clones per sample) was performed on the 10 subjects in cohort A with HCV RNA levels of $>1,000$ IU/ml at the end of treatment (day 7) to assess their quasispecies composition and dynamics at baseline, day 4 (end of monotherapy), and day 7 (end of combination therapy) (Fig. 3).

Baseline samples from two of the three patients in cohort A1 that had an HCV RNA level of $>1,000$ IU/ml at day 7 contained at frequencies of 1 to 2% minority variants that confer resistance to danoprevir or to another PI but not to mericitabine. The observed PI-resistant variants were not enriched during mericitabine monotherapy or mericitabine-danoprevir dual therapy (Fig. 3).

Baseline samples from five of the seven patients in cohort A2 that had an HCV RNA level of $>1,000$ IU/ml at day 7 contained at

low frequencies (1 to 2%) minority variants that conferred resistance to danoprevir or to another PI but not to mericitabine. These variants were not enriched during danoprevir monotherapy or mericitabine-danoprevir dual therapy. In the two patients with no PI-resistant variants at baseline, low-frequency PI resistance variants were observed at day 4 and/or day 7 (Fig. 3).

No mericitabine-resistant variants were selected during monotherapy. Moreover, the dynamics of the NS3 variants detected within patients' quasispecies at baseline were similar after danoprevir or mericitabine monotherapy, suggesting that they arose as the result of random mutagenesis rather than being driven by drug-associated selective pressure.

Drug susceptibility studies of variants containing PI-resistant amino acid substitutions. Susceptibility to danoprevir and telaprevir was assessed in 41 replicon clones isolated from the quasispecies of patients included in this resistance analysis. Eighteen of 41 clones represented consensus sequences, and the sequences of the remaining 23 clones differed from the consensus sequence by having amino acid substitutions that either were known to confer PI resistance or occurred at residues that are associated with PI resistance (and for which the effect on PI susceptibility has not yet been described) (Table 2). Most clones that contained PI resistance mutations had a lower replication capacity than the corresponding consensus clone.

Amino acid changes at residue V36 (V36A or V36M) did not confer resistance to danoprevir (1.7- and 0.9-fold increases in EC_{50} s versus the EC_{50} for the Con1 reference control). V36M confers low-level resistance to telaprevir (5-fold increase in EC_{50} versus that for the Con1 reference strain) (42). The mutation Q41R conferred resistance to both danoprevir (50- to 100-fold increases

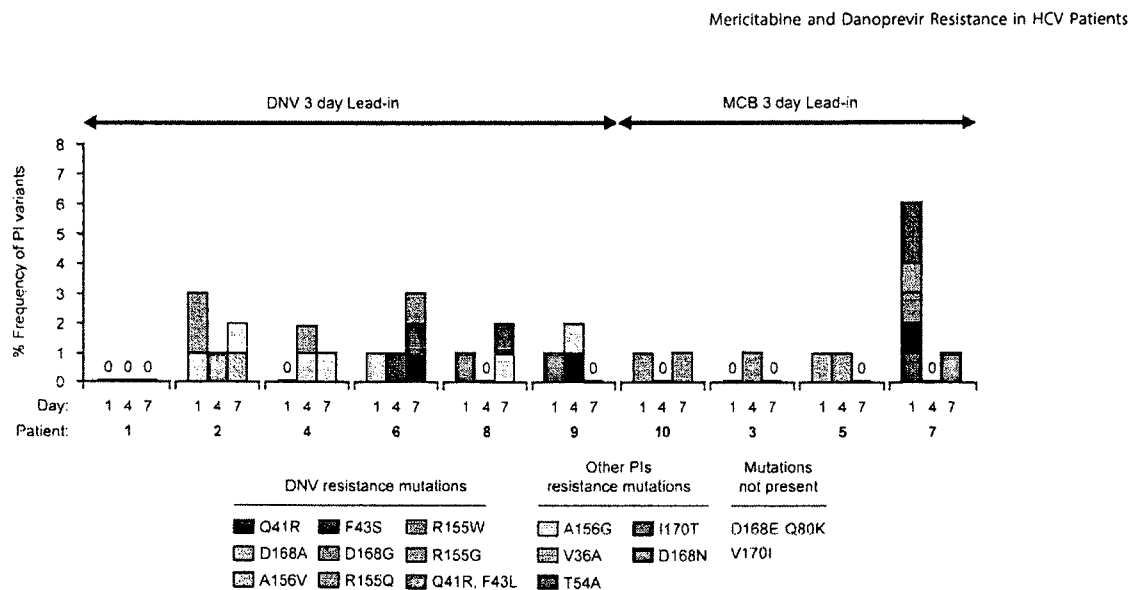


FIG 3 Presence and viral dynamics of low-frequency PI-resistant variants in cohort A patients with an end-of-treatment serum HCV RNA level of $\geq 1,000$ IU/ml. DNV, danoprevir; MCB, mericitabine.

in EC₅₀s versus the EC₅₀ for the consensus clone from the same sample) and telaprevir (<12-fold increases).

Samples with changes at residue 43 were evaluated for their susceptibility to danoprevir. These included F43Y/A156V, which was observed in 1.2% of clones from patient 19 on day 3; Q41R/F43L, which was observed in 1.1% of clones from patient 2 on day 4; and F43S, which was observed in 1% of clones from patient 84, who experienced viral breakthrough on study day 15. The Q41R/F43L variant was the only one that replicated *in vitro* and subsequently showed an increase in EC₅₀ similar to that observed for the mutant with a single mutation, Q41R, suggesting that F43L does not affect susceptibility to danoprevir.

Effects of substitutions at amino acid Q80 on danoprevir susceptibility depended on the genetic context. Q80K conferred low-level resistance to danoprevir (10-fold) but not to telaprevir in a clone from patient 19 (C06, genotype 1a) (Table 2). Three other genotype 1a isolates that contained predominantly Q80K (from patients 42 and 52) or a Q80K/Q mixture (from patient 71) were evaluated (as a pool of 96 clones) and showed no significant reduction in susceptibility to danoprevir (EC₅₀s, 0.28 ± 0.05 nM [2-fold increase compared to a Con1 EC₅₀ of 0.14 ± 0.002 nM], 0.29 ± 0.016 nM [2-fold increase], and 0.09 ± 0.001 nM [0.64-fold increase], respectively) (Table 2).

Substitutions at residue 155 were evaluated in different isolates and therefore different genetic contexts. R155K-containing clones showed high-level resistance (>800-fold) to danoprevir, medium-level resistance (~15-fold) to telaprevir, and also a reduced replication capacity. One R155K-containing clone that had an additional mutation at position 106 (L to P), R155G-containing variants from two genotype 1a-infected patients, and several R155Q-containing clones could not be evaluated because of low/no replication *in vitro*. Reduced susceptibility to danoprevir (27-fold) but not to telaprevir was observed in a variant containing R155W.

The A156G substitution did not confer resistance to either danoprevir or telaprevir; in contrast, A156V conferred high-level resistance to danoprevir and to telaprevir (>100-fold and >1,000-fold increases in EC₅₀s, respectively) (Table 2).

Amino acid change I/V170I was observed in the on-treatment population sequence of one patient in cohort A and was found at a low prevalence in the quasispecies of four baseline samples (Table 1 and 2). This substitution had no significant effect on susceptibility to danoprevir (2.6-fold decrease versus the consensus clone; Table 2). The substitution I170T was observed in one genotype 1a genetic context and conferred low-level resistance (6-fold) to telaprevir but not to danoprevir.

DISCUSSION

Interferon-free therapy could become a new option for the treatment of chronic hepatitis C virus infection. Recent clinical studies in small cohorts of HCV-infected patients have shown that high sustained virological response rates can be achieved using combinations of two direct-acting antiviral agents (DAAs) (2, 8, 23). The success of interferon-free regimens hinges on two major elements that enable a durable response: profound viral suppression and a high barrier to resistance. Resistance to danoprevir in patients receiving danoprevir monotherapy is conferred by the mutation NS3 R155K (22). Low-level resistance to mericitabine is conferred through the NS5B mutation S282T (1).

The INFORM-1 study showed that an interferon-free regimen of two DAAs (mericitabine and danoprevir) could produce a rapid and continual decline in HCV RNA through 13 days of treatment with no resistance-related viral breakthrough, as assessed by population and clonal sequence and drug susceptibility analysis (6). In contrast, danoprevir monotherapy was associated with viral load breakthrough and resistance selection by day 7 in five of eight patients (22). This suggests that mericitabine effectively suppressed the emergence of danoprevir-resistant virus during up to 13 days of treatment, even in regimens that contained suboptimal doses of either mericitabine, danoprevir, or both drugs. It is acknowledged that these results could differ with longer treatment durations if clearance of the danoprevir-resistant virus by mericitabine is not maintained.

Viral breakthrough due to selection of drug-resistant HCV has been detected within as little as 3 days of the start of treatment with telaprevir monotherapy (5, 13, 32–35), and resistant HCV vari-

TABLE 2 Comparative susceptibility to danoprevir and telaprevir of HCV-containing NS3 mutations in baseline samples from patients in cohorts A to D

Strain or site(s) of polymorphism and patient sample (HCV genotype), day	Clone no.	Sequence/difference from consensus (amino acid)	Frequency in viral population ^a	Replication	Danoprevir		Telaprevir	
					EC ₅₀ ± SEM (nM)	Fold shift ^b	EC ₅₀ ± SEM (μM)	Fold shift ^b
Con1				1	0.19 ± 0.020 (16) ^c		0.13 ± 0.025 (15)	
Residue 170								
1 (1a), day 1	A04	Consensus	58.2	0.017 ± 0.008 (2)	0.11 ± 0.013 (2)	1	0.41 ± 0.041 (2)	1
	A10	V170I	9.9	0.031 ± 0.012 (2)	0.12 ± 0.008 (2)	1.10	0.21 ± 0.023 (2)	0.51
1 (1a), day 7	A02	Consensus	32.9	0.097 ± 0.022 (4)	0.25 ± 0.033 (2)	1	0.10 ± 0.025 (2)	1
	A06	V170I	14.5	0.046 ± 0.003 (2)	0.12 ± 0.029 (3)	0.48	0.053 ± 0.004 (2)	0.53
2 (1a), day 1	A04	Consensus	33.7	0.24 ± 0.040 (2)	0.17 ± 0.041 (2)	1	0.21 ± 0.019 (2)	1
	A02	I170V	3.6	0.061 ± 0.023 (2)	0.10 ± 0.006 (2)	0.58	0.27 ± 0.022 (2)	1.28
5 (1a), day 1	A02	Consensus	76.2	0.028 ± 0.003 (4)	0.080 ± 0.020 (3)	1	0.022 ± 0.001 (2)	1
	D03	I170V	2.5	0.022 ± 0.004 (4)	0.11 ± 0.016 (2)	1.37	0.039 ± 0.010 (2)	1.77
7 (1a), day 1	A03	Consensus	56.6	0.038 ± 0.009 (4)	0.12 ± 0.034 (3)	1	0.060 ± 0.015 (2)	1
	C11	I170T	1.3	0.006 ± 0.001 (6)	0.32 ± 0.027 (5)	2.66	0.35 ± 0.030 (3)	5.83
Residue 155								
2 (1a), day 7	D06	R155G	1.1	No replication				
7 (1a), day 1	F04	R155G	1.3	No replication				
10 (1b), day 1	A02	Consensus	28.0	0.22 ± 0.030 (2)	0.11 ± 0.010 (4)	1	0.096 ± 0.011 (2)	1
	A12	R155W	1.2	0.001 ± 0.00 (4)	3.06 ± 0.21 (2) ^d	27.81	0.20 ± 0.033 (2) ^d	2.08
10 (1b), day 7	A01	Consensus	68.0	0.090 ± 0.024 (3)	0.12 ± 0.012 (2)		0.14 ± 0.004 (2)	
	B04	R155Q	1.0	0.001 ± 0.00 (2) ^e				
20 (1a), day 1	A05 ^f	Consensus (155K)	62.2	0.020 ± 0.001 (3)	>100 (2)	>833	0.78 ± 0.11 (2)	14.44
	E07 ^g	K155R	1.1	0.038 ± 0.006 (3)	0.12 ± 0.017 (3)	1	0.054 ± 0.006 (2)	1
20 (1a), day 4	A02 ^f	Consensus (155K)	79.3	0.005 ± 0.001 (3)	>100 (2)		0.90 ± 0.088 (2)	
	A08	L106P, R155K	1.1	No replication				
20 (1a), day 10	A01 ^g	K155R	29.9	0.080 ± 0.022 (3)	0.11 ± 0.029 (3)	1	0.043 ± 0.005 (3)	1
	A05 ^f	Consensus (155K)	47.1	0.013 ± 0.002 (3)	>100 (2)	>909	0.63 ± 0.084 (3)	14.65
20 (1a), day 14	A01 ^g	Consensus (155R)	48.2	0.063 ± 0.025 (3)	0.11 ± 0.033 (3)	1	0.051 ± 0.005 (3)	1
	A04 ^f	R155K	36.4	0.019 ± 0.002 (2)	>100 (2)	>909	0.72 ± 0.11 (3)	14.11
Residues D168E and Q80K								
19 (1a), day 1	A02 ^h	Consensus (168E)	64.8	0.38 ± 0.141 (2)	9.58 ± 1.69 (2)	53.22	0.041 ± 0.005 (2)	1.36
	C06	Q80K, E168D, V170I	1.1	0.38 ± 0.100 (2)	1.81 ± 0.12 (2)	10.05	0.068 ± 0.009 (2)	1.21
	H11	E168D, V170I	1.1	0.22 ± 0.082 (2)	0.18 ± 0.052 (2)	1	0.056 ± 0.011 (2)	1
19 (1a), day 3	A01 ^h	Consensus (168E)	87.8	0.30 ± 0.13 (2)	18.76 ± 1.671 (2)		0.12 ± 0.011 (2)	
	C07	F43Y, A156V	1.2	No replication				
Residues known to confer resistance to danoprevir <i>in vitro</i>								
2 (1a), day 7	A01	Consensus	71.9	0.026 ± 0.004 (4)	0.14 ± 0.022 (5)	1	0.23 ± 0.037 (2)	
	H12	A156G	1.1	0.009 ± 0.004 (2)	0.17 ± 0.026 (2)	1.21	0.34 ± 0.093 (3)	1.47
5 (1a), day 1	A02	Consensus	76.2	0.028 ± 0.003 (4)	0.080 ± 0.020 (3)	1	0.022 ± 0.001 (2)	
	H07	A156V	1.2	0.008 ± 0.004 (4)	10.5 ± 2.96 (3)	131.25	>30 (2)	>1,363
6 (1a), day 7	A02	Consensus	51.7	0.023 ± 0.007 (2)	0.12 ± 0.011 (2)	1	0.082 ± 0.012 (2)	1
	B12	Q41R	1.1	0.014 ± 0.006 (2)	11.14 ± 0.38 (2)	92.83	1.04 ± 0.022 (2)	12.70
9 (1a), day 4	A04	Consensus (V36 M)	69.7	0.035 ± 0.017 (2)	0.17 ± 0.021 (2)	1	0.67 ± 0.015 (2)	1
	C08	Q41R, V36 M	1.1	0.005 ± 0.002 (2)	8.48 ± 1.31 (2)	49.90	7.46 ± 1.442 (2)	11.13
11 ⁱ (1b), day 15	A08	Consensus	57.9	0.020 ± 0.008 (2)	0.17 ± 0.023 (2)		0.027 ± 0.008 (2)	
	C10	F43S	1.1	No replication				
19 (1a), day 4	A07	Consensus	36.8	0.16 ± 0.076 (2)	0.13 ± 0.019 (2)	1	0.16 ± 0.031 (2)	1
	D09	Q41R, F43L	1.1	0.004 ± 0.001 (2)	8.28 ± 1.38 (2)	63.70	0.71 ± 0.12 (2)	4.75
Residue 36, 2 (1a), day 1	A04	Consensus	33.7	0.24 ± 0.040 (2)	0.17 ± 0.041 (2)	1	0.21 ± 0.019 (2)	1
	E02	V36A	1.1	0.068 ± 0.003 (2)	0.32 ± 0.047 (2)	1.88	0.52 ± 0.094 (2)	2.47

^a Percentage out of total number of sequenced clones within each sample. The total number of sequenced clones varied from 76 to 94 per sample.

^b Fold shift was calculated using the variant that did not contain any resistance mutation.

^c Values in parentheses are numbers of patients.

^d EC₅₀ determination is approximate, as inhibition curves did not reach 100%.

^e Level of replication too low for EC₅₀ determination.

^f Identical clones within a patient.

^g Identical clones within a patient.

^h Identical clones within a patient.

ⁱ The patient that experienced viral breakthrough while on dual therapy (8).

ants have emerged within 8 days of the initiation of treatment with telaprevir, peginterferon alfa-2a (40KD), and ribavirin triple-combination therapy (16). For this reason, the absence of protease-resistant variants after 13 days of combination therapy in this trial suggests that mericitabine has the potential to prevent or delay the emergence of PI-resistant variants.

The high viral replication rate and the nucleotide misincorporation rate of the HCV NS5B polymerase result in the formation of a viral quasispecies, resulting in a situation in which many viral sequence variants, including drug-resistant variants, can preexist in untreated patients. DAA-resistant virus was detected in un-

treated patients by population sequencing of NS3- and NS5B-coding sequences. The most frequent mutations were associated with resistance to nonnucleoside polymerase inhibitors (data not shown). Less frequent were mutations associated with resistance to PIs, while the mutation S282T, which confers *in vitro* resistance to mericitabine, PSI-7977, and IDX-184, was not detected in any sample, similar to previous reports (14, 19, 39, 40).

This study provides a comprehensive clonal assessment of the prevalence of minority variants affecting PI susceptibility (a total of 2,937 clonal variants were analyzed) and the dynamics of such variants within the quasispecies upon treatment.

The NS5B S282T variant was not detected by population or clonal analysis in any samples collected at baseline or during treatment, consistent with previous studies where patients received mericitabine alone for 2 weeks or in combination with peginterferon alfa-2a (40KD) plus ribavirin for up to 24 weeks (12, 19, 21, 29). Position 282 of the NS5B polymerase is located near the active binding site and is highly conserved across all HCV genotypes. If the serine at this position is required for polymerase function, this would explain the low *in vitro* replicative capacity of S282T-containing mutant replicons (1, 27) and could explain the high barrier to resistance observed *in vivo*.

While it has been shown that DAA-resistant variants may exist at low frequencies within the quasispecies of untreated patients, their clinical relevance has not yet been determined. Variants containing danoprevir resistance mutations were detected as minority species at baseline in 4 out of 12 studied patients (10 in cohort A, 1 in cohort B, 1 in cohort E). These variants were not enriched during treatment, suggesting that the slower kinetics of plasma HCV RNA reduction in patients in cohort A were not associated with the presence and/or selection of virus resistant to danoprevir and/or mericitabine. The dynamics of the minority PI-resistant variants at baseline and throughout treatment were similar after danoprevir or mericitabine monotherapy. The low frequency of resistant variants was, therefore, likely due not to drug pressure but, rather, to random mutagenesis caused by the error-prone nature of the NS5B polymerase. In addition, relative fitness and immune pressure may determine the relative proportion of minority variants within a given patient's quasispecies (9). As an example of intrinsic quasispecies dynamics, the NS3 R155K substitution was predominant at baseline (98% of clones) in a patient who received placebo (patient 20) and then reverted to wild type by day 13 (55% wild type), likely because of the lower fitness of this variant compared with wild type. To address the potential relevance of minority variants on susceptibility to PIs, extensive phenotypic studies were performed on individual variants that either contained amino acid substitutions known to confer resistance to PIs or had variations that occurred at residues known to be associated with resistance to PIs but that have not yet been described. While certain amino acid changes, such as NS3 Q41R, affected susceptibility to both linear (i.e., telaprevir) and macrocyclic (i.e., danoprevir) PIs, other changes affected susceptibility to specific drugs (V36M, R155W, I170T, or Q80K). Importantly, all patients achieved a continuous viral load decline, suggesting that minority species with reduced drug susceptibility could be effectively suppressed by the combination of mericitabine and danoprevir.

In summary, the present study showed that minority variants resistant to PIs exist within quasispecies of treatment-naïve patients as either predominant or minority species. New NS3 amino acid substitutions affecting susceptibility to telaprevir (V36M, I170T) or danoprevir (R155W, Q80K), or both drugs (Q41R), were observed in individual minority variant clones and were characterized for the first time. Importantly, this study shows that the genetic context can affect susceptibility to PIs, highlighting the importance of phenotypic studies in resistance monitoring. Minority PI-resistant variants that are present at baseline can be effectively suppressed by the inclusion of the nucleoside analog mericitabine in a combination regimen and are unlikely to be clinically relevant. This study shows that HCV RNA levels of >1,000 IU/ml at day 13 in 14 patients in the INFORM-1 study were not due to the selection of resistance to mericitabine or dano-

previr. This suggests that the combination of a PI with a nucleoside polymerase inhibitor that has a high barrier to resistance, such as mericitabine, has the potential to reduce or prevent the emergence of clinically significant resistance to PIs even when PI-resistant variants are present at baseline.

The primary limitation of this study is the short duration of combination therapy, which likely explains why not all patients achieved HCV RNA levels below 1,000 IU/ml at the end of dual-combination therapy. Longer-duration studies are needed to confirm that mericitabine can prevent the emergence of resistance and to determine the durability of resistance suppression when mericitabine is combined with a PI or other DAA. These encouraging results support further studies of this dual-combination interferon-free regimen.

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