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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.

**Trámite: 11**
**Evento: 378**
**Actuación: 523**

Re: P1.-**RIBOSCIENCE LLC.**-Solicitud de Patente de Invención en Fase Nacional PCT para **"DERIVADOS NUCLEÓSIDOS CON SUSTITUCIÓN 2',4'-DIFLUORO-2'-METILO COMO INHIBIDORES DE LA REPLICACIÓN DEL ARN DEL VHC"**.-Clase **C07H 19/06**.-Expediente número **14-106.193.-**

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista de 21 de Abril de 2015.

2. Con el fin de responder a las objeciones expuestas por el Señor Examinador bajo los acápites de Excepciones a la Patentabilidad, Reivindicaciones relativas a un Uso o a un segundo uso, Concisión del capítulo reivindicatorio, Novedad y Nivel Inventivo, atentamente me permito presentar una serie de argumentos técnicos, así como una serie de modificaciones en el capítulo reivindicatorio de la solicitud de patente en cuestión, por medio de lo cual se superan completamente dichas objeciones.

3. **EXCEPCIÓN A LA PATENTABILIDAD**

El señor Examinador ha objetado las reivindicaciones 19 y 20 en vista de que éstas no son patentables en virtud del artículo 20 de la Decisión 486 de 2000.

Atendiendo a lo anterior, el solicitante ha decidido eliminar dichas reivindicaciones 19 y 20, con lo cual se supera completamente al objeción realizada por el señor Examinador a éste respecto.

4. **REIVINDICACIONES RELATIVAS A UN USO O A UN SEGUNDO USO**

El señor Examinador ha objetado las reivindicaciones 15, 17 y 18 en vista de que éstas no son patentables en virtud del artículo 14 de la Decisión 486 de 2000.

Atendiendo a lo anterior, el solicitante ha decidido eliminar dichas reivindicaciones 15, 17 y 18, con lo cual se supera completamente al objeción realizada por el señor Examinador a éste respecto.

## 5. CLARIDAD Y CONCISIÓN DEL CAPÍTULO REIVINDICATORIO

El señor Examinador aduce que la reivindicación 1 carece de concisión y claridad porque: *"...emplea términos tales como haloalquilo inferior, arilo, alquilo inferior, alquinilo inferior, alcoxi inferior, halo, acilamino, aril-alquilo inferior, fenil-alquilo inferior, ya que con tales definiciones se pueden presentar diferentes opciones de posibles sustituciones que no permiten reconocer el objeto que se pretende proteger. Por ejemplo cuando el término arilo o alquilo no define el número de átomos que lo conforman. Por tal motivo se obtienen un número indefinido de compuestos que no encuentran soporte en la descripción."*.

Frente a lo anterior me permito manifestar que el solicitante ha decidido reemplazar los términos haloalquilo inferior, alquilo inferior, alquinilo inferior, alcoxi inferior, aril-alquilo inferior y fenil-alquilo inferior, por los nuevos términos haloalquilo$C_{1-6}$, alquilo$C_{1-6}$, alquinilo$C_{1-6}$, alcoxi$C_{1-6}$, acilamino, aril-alquilo$C_{1-6}$ y fenil-alquilo$C_{1-6}$.

De otro lado, con respecto al término *'arilo'* atentamente me permito aclarar que en la reivindicación 1 actualmente presentada se establece que dicho término representa un grupo seleccionado entre fenilo o naftilo, cualquiera de los dos sin substituir o sustituido con uno o más de entre alquilo$C_{1-6}$, alquenilo$C_{2-6}$, alquinilo$C_{2-6}$, alcoxi$C_{1-6}$, halo, haloalquilo$C_{1-6}$, $-N(R^{1a})_2$, acilamino, $-SO_2N(R^{1a})_2$, $-COR^{1b}$, $-SO_2(R^{1c})$, $-NHSO_2(R^{1c})$, nitro o ciano. Lo anterior, tal y como lo apreciará el señor Examinador, representa un grupo conciso de posibles significados para dicho grupo *'arilo'*.

Visto lo anterior me permito manifestar que el nuevo capítulo reivindicatorio de la solicitud de patente en cuestión cumple a cabalidad con los requisitos de patentabilidad establecidos en el Artículo 30 de la Decisión 486 de 2000, toda vez que es claro, conciso y encuentra pleno soporte en la descripción de la invención.

## 6. NOVEDAD DE LA SOLICITUD DE PATENTE

El señor Examinador asevera que las reivindicaciones 1 a 10, 12, 13 y 16 no cumplen con el requisito de novedad frente a las enseñanzas del documento US2010/016251 (D1).

En respuesta a lo anterior, atentamente me permito plantear los siguientes argumentos técnicos:

- Inicialmente el señor Examinador afirma que:

  *"De acuerdo con la tabla anterior, donde se comparan las características técnicas de la reivindicación 1 con el documento D1, se concluye que los compuestos aquí definidos no cumplen con el requisito de novedad puesto que todas las características técnicas esenciales ya se encuentran reveladas en dicho documento del estado del arte. Por lo tanto la reivindicación 1 no es nueva, o carece de novedad.",*

  Pero más adelante el mismo señor Examinador también afirma lo siguiente:

  *"La diferencia entre los compuestos de la invención y los revelados en D1 consiste en la sustitución de* $R^5$ *cuando es* $P(=O)(OR1)(NR_4R_7)$*.".*

Lo anterior, por supuesto, representa una contradicción del señor Examinador en su Concepto Técnico, hecho que no permite elucidar con claridad las razones en las que se basa para objetar la novedad de la solicitud de patente en cuestión.

- Y es que en efecto, una comparación cuidadosa entre la estructura química de los compuestos revelados en la presente invención y aquellos divulgados en el documento D1 permite establecer que existen claras diferencias entre estos.

- En este mismo sentido, el Reporte preliminar internacional frente a la patentabilidad señala que, en efecto, en el documento D1 no se divulgan de manera explícita aquellos compuestos químicos que caen dentro del alcance de la solicitud de patente en cuestión, reconociendo por lo tanto la novedad de la solicitud.

Visto lo anterior, atentamente solicito al señor Examinador la reconsideración de las objeciones realizadas a éste respecto.

## 7. NIVEL INVENTIVO DE LA SOLICITUD DE PATENTE

Finalmente, el señor Examinador aduce que las reivindicaciones 1 y 14 no cumplen con el requisito de nivel inventivo frente a las enseñanzas del documento D1, señalando en éste caso que las diferencias existentes no conceden un efecto técnico inesperado y que las modificaciones en los sustituyentes de compuestos conocidos son posibilidades plenamente conocidas para la persona del oficio normalmente versada en la materia.

En respuesta a lo anterior y con el ánimo de demostrar el nivel inventivo que tiene la materia revelada en la presente invención frente a las enseñanzas del arte anterior, y particularmente frente a aquellas enseñanzas del documento D1, atentamente me permito plantear lo siguiente:

- Inicialmente me permito resaltar que la realización de una búsqueda de anterioridades tendiente a ubicar enseñanzas previas con respecto a compuestos químicos análogos de nucleósidos que tengan una combinación de sustituyentes 2'-deoxi-2',4'-difluoro-2'-C-metilo, tal y como ocurre en la solicitud de patente en cuestión, arroja que en el estado de la técnica no existen y tampoco se sugieren éste tipo de compuestos, lo cual, sumado al hecho de que los análogos de nucleósidos para el tratamiento del virus de la hepatitis C (VHC) comenzaron a desarrollarse desde hace más de 20 años, sugiere drásticamente que los compuestos revelados en la presente invención de ninguna manera resultan obvios para la persona medianamente versada en la materia.

- Ahora bien, con respecto al argumento del señor Examinador de acuerdo con el cual *"El documento D1 ya realiza sugerencias adecuadas para llegar a la presente invención, particularmente revela compuestos que son estructuralmente equivalentes a los compuestos de la presente invención, los cuales también son inhibidores del VHC y cuya estructura principal contempla derivados nucleósidos con sustitución 2'-4'-difluoro-2'-metilo"*, atentamente me permito resaltar ante ese Despacho que el área de síntesis química de nucleósidos es plenamente conocido que cualquier cambio sobre el anillo de la ribosa puede traducirse de manera impredecible en cambios en su actividad biológica.

En este sentido, el siguiente aparte del artículo científico *Antiviral Research (2013) 100, 500-519* (correspondiente al Anexo II del presente memorial) señala las razones técnicas por las cuales es falso aseverar que los efectos de modificaciones estructurales ligeras que son típicas en la química tradicional también aplican para aquellas modificaciones realizadas sobre la estructura de nucleósidos:

*"A pesar de que es conceptualmente sencillo, existen varios retos clave en el desarrollo de NIs (inhibidores análogos de nucleósido/nucleótido) para terapia. Primero, es frecuentemente imposible predecir la relación estructura-actividad de los compuestos, debido a que se requieren varias etapas enzimáticas para convertirlos a tri-fosfatos activos (Stein y Moore, 2001). Esto genera un reto para los planteamientos de la química medicinal clásica con el objetivo de mejorar la*

*potencia. Por lo tanto, los NIs potentes son descubiertos frecuentemente por serendipia. Segundo, la toxicidad de los análogos de nucleósido es impredecible y generalmente se pierde in vitro. Aunque la toxicidad mitocondrial, tal como la inhibición de la polimerasa gamma, han estado implicadas en muchos Nis antivirales, especialmente en aquellos para el tratamiento de infecciones con VHC-1, existen otras toxicidades asociadas con Nis (Dagan y compañeros, 2002). Por lo tanto, los estudios toxicológicos en animales son frecuentemente necesarios para evaluar los potenciales efectos laterales de esta clase de compuestos, lo cual es frecuetemente costoso en términos de requerimientos de compuestos y animales.".*

- Adicionalmente me permito señalar que, por ejemplo, la adición de un sustituyente en la posición 4' de un análogo de nucleósido que ya cuenta con sustituyentes en la posición 2' del anillo de ribosa puede variar significativamente la actividad biológica y llevar a consecuencias inesperadas, y de acuerdo con lo señalado en el artículo científico *Antiviral Res. 2014, February, 102: 119-147* (correspondiente al Anexo III del presente memorial) los compuestos usualmente conocidos por ser particularmente activos contra la polimerasa del VHC son compuestos nucleósidos sustituidos en las posiciones 2' y 4'.

Sin embargo, también ha sido reconocido que dichos compuestos típicamente corresponden en realidad a compuestos sustituidos en la posición 2' o en la posición 4', pero no en ambas:

*"A la fecha, muchos de los análogos de nucleósidos reportados que inhiben la polimerasa del VHC tienen modificaciones en la posición 2' o 4' del azúcar [1-12]." Antiviral Chemistry & Chemotherapy 2009, 20: 99-106* (correspondiente al anexo IV del presente memorial).

- Así mismo, y con relación específica a las enseñanzas del documento D1, considero relevante destacar que de ninguna manera resulta obvio la actividad biológica de un compuesto análogo de nucleósido 2'-fluoro-4'-fluoro con base en la actividad mostrada por los análogos de nucelósido 2'-fluoro-2'-C-metilo divulgados en dicho documento D1, toda vez que de acuerdo con lo publicado por los autores del artículo (Antiviral Chemistry & Chemotherapy, 2009, 20: 99-106) luego de intentar combinar análogos de nucleósidos 4'-sustituidos y 2'-metil sustituidos para el tratamiento del VHC en la forma de nucleósidos de citidina y uridina 4'-azido-2'-C-metil el resultado logrado fue que no todos los análogos de nucleósido obtenidos son activos contra el virus de la hepatitis C.

Consecuentemente, es claro que las enseñanzas del estado de la técnica en realidad alejarían a la persona medianamente versada en la materia de la posibilidad de suponer que llevar a cabo sustituciones en las posiciones 2' y 4' de análogos de nucleósido permitirían lograr compuestos con actividad biológica contra el VHC.

- Por último, considero relevante destacar ante ese Despacho que es completamente inesperado que un análogo de uridina (de conformidad con la estructura química de los compuestos revelados en la solicitud de patente en cuestión) tenga una mayor actividad frente al VHC en comparación con un análogo de citidina, siendo ésta una de las principales diferencias frente a las enseñanzas del documento D1 porque dicha anterioridad de ninguna manera está limitada a análogos de uridina, sino que incluye todas las bases, tanto purinas como pirimidinas.

Visto lo anterior me permito manifestar que la materia revelada en la solicitud de patente en cuestión presenta un inequívoco carácter inventivo frente a las enseñanzas del estado de la técnica anterior, cumpliendo por lo tanto a cabalidad con las disposiciones de patentabilidad establecidas en el artículo 18 de la Decisión Andina 486 de 2000.

## 8. CONCESIÓN DE LA PATENTE EN ESTADOS UNIDOS

Aparte de todo lo mencionado hasta el momento, atentamente me permito señalar ante ese Despacho que la Oficina marcas y patentes de Estados Unidos ha reconocido los méritos de patentabilidad de la materia técnica en cuestión *(El anexo V del presente recurso corresponde a una copia de la notificación de la concesión de la patente)*.

Ahora bien, ante todo es preciso señalar que con la información mencionada anteriormente de ninguna manera pretendo desconocer la independencia de la que goza la Superintendencia de Industria y Comercio al momento de analizar los méritos de patentabilidad de una solicitud, de acuerdo con las disposiciones del Convenio de París para la protección de la propiedad industrial.

Sin embargo, sí considero de gran importancia resaltar el hecho de que la materia técnica revelada en la solicitud de patente en cuestión corresponde a materia que ha sido catalogada como novedosa, inventiva e industrialmente aplicable por los Examinadores de la Oficina de marcas y patentes de Estados Unidos, hecho que evidentemente representa un claro indicio de que la materia técnica revelada en la solicitud de patente en estudio cumple a cabalidad con los requisitos de patentabilidad establecidos en la Decisión Andina 486 de 2000.

9. **CONCLUSIÓN**

De conformidad con las modificaciones realizadas en el capítulo reivindicatorio y teniendo en cuenta los argumentos técnicos proporcionados en el presente escrito, es claro que los documentos del arte previo señalados por ese Despacho en el Concepto Técnico en cuestión de ninguna manera pueden afectar la novedad y el nivel inventivo de los compuestos químicos revelados en la solicitud de patente en cuestión.

10. Anexo me permito presentar:

i) Recibo número **15-0073500**, expedido por la Superintendencia de Industria y Comercio, que acredita el pago por las reivindicaciones **11** a **15**;

ii) Copia del nuevo capítulo reivindicatorio, el cual está compuesto por 15 reivindicaciones y reemplaza el documento actualmente presentado;

iii) Copia del Artículo Científico *Antiviral Research (2013) 100, 500-519;*

iv) Copia del Artículo Científico *Antiviral Res. 2014, February, 102: 119-147;*

v) Copia del Artículo Científico *Antiviral Chemistry & Chemotherapy 2009, 20: 99-106;*

vi) Copia del documento expedido por la USPTO en relación con la solicitud de patente equivalente a aquella analizada en el presente memorial.

11. En vista de lo anterior, ruego a usted proceder a estudiar nuevamente la solicitud a la luz de los argumentos presentados y conceder el privilegio de patente solicitado.

Señor Superintendente,

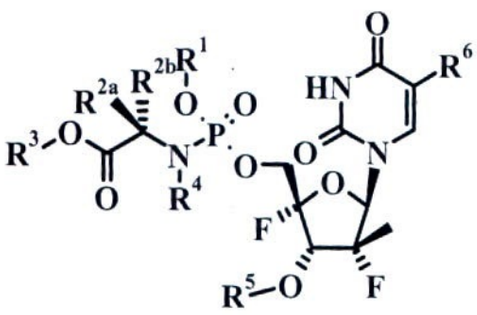
ALICIA LLOREDA RICAURTE
T.P. 53.215

Riboscience LLC
Exp. 14-106.193



## REIVINDICACIONES

Compuesto de fórmula I:



I

en la que:

$R^1$ es H, haloalquilo$C_{1-6}$ o arilo, en el que arilo es fenilo o naftilo no sustitupido o sustituido con uno o más de entre alquilo$C_{1-6}$, alquenilo$C_{2-6}$, alquinilo$C_{2-6}$, alcoxi$C_{1-6}$, halo, haloalquilo$C_{1-6}$, -$N(R^{1a})_2$, acilamino, -$SO_2N(R^{1a})_2$, -$COR^{1b}$, -$SO_2(R^{1c})$, -$NHSO_2(R^{1c})$, nitro o ciano,

cada $R^{1a}$ es independientemente H o alquilo$C_{1-6}$,

cada $R^{1b}$ es, independientemente, -$OR^{1a}$ o -$N(R^{1a})_2$,

cada $R^{1c}$ es alquilo$C_{1-6}$,

$R^{2a}$ and $R^{2b}$ son (*i*) independientemente H, alquilo$C_{1-6}$, -$(CH_2)_rN(R^{1a})_2$, hidroxialquilo$C_{1-6}$, -$CH_2SH$, -$(CH_2)S(O)_pMe$, -$(CH_2)_3NHC(=NH)NH_2$, (1*H*-indol-3-il)metilo, (1*H*-indol-4-il)metilo, -$(CH2)_mC(=O)R^{1b}$, arilo y aril-alquilo$C_{1-6}$, en el que el arilo puede sustituirse opcionalmente con uno o más de entre hidroxi, alquilo$C_{1-6}$, alcoxi$C_{1-6}$, halo, nitro o ciano, (*ii*) $R^{2a}$ es H y $R^{2b}$ y $R^4$ conjuntamente forman $(CH_2)_3$; (*iii*) $R^{2a}$ y $R^{2b}$ conjuntamente forman $(CH_2)_n$, o (*iv*) $R^{2a}$ y $R^{2b}$ son ambos alquilo$C_{1-6}$,

$R^3$ es H, alquilo$C_{1-6}$, haloalquilo$C_{1-6}$, fenilo o fenil-alquilo$C_{1-6}$,

$R^4$ es H, alquilo$C_{1-6}$ o $R^{2b}$ y $R^4$ conjuntamente forman $(CH_2)_3$,

$R^5$ es H, $C(=O)R^{1c}$, $C(=O)R^{1b}$ , $P(=O)(OR^1)(OR^{1a})$ o $P(=O)(OR^1)(NR^4R^7)$,

$R^6$ es H, metilo o halo,

$R^7$ es $C(R^{2a}R^{2b})COOR^3$

m es 0 a 3,

n es 4 ó 5,

p es 0 a 2, y

r es 1 a 6,

o una sal farmacológicamente aceptable de los mismos.

2. Compuesto según la reivindicación 1, en el que $R^4$ es H.

3. Compuesto según la reivindicación 2, en el que $R^6$ es H o Br.

4. Compuesto según la reivindicación 3, en el que $R^1$ es naftilo o fenilo.

5. Compuesto según la reivindicación 4, en el que $R^{2a}$ es H.

6. Compuesto según la reivindicación 5, en el que $R^{2b}$ es metilo.

7. Compuesto según la reivindicación 6, en el que $R^3$ es isopropilo.

8. Compuesto según la reivindicación 7, en el que $R^5$ es H.

9. Compuesto según la reivindicación 7, en el que $R^5$ es $C(=O)R^{1c}$.

10. Compuesto según la reivindicación 9, en el que $R^{1c}$ es etilo.

11. Compuesto según la reivindicación 7, en el que $R^5$ es $P(=O)(OR^1)(NR^4R^7)$.

12. Compuesto según la reivindicación 11, en el que $R^1$ es naftilo.

13. Compuesto según la reivindicación 12, en el que $R^4$ es H y $R^7$ es $CH(CH_3)C(=O)OCH(CH_3)_2$.

14. Compuesto seleccionado entre el grupo que consiste de:

isopropil-éster de ácido (S)-2-{[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-fenoxi-fosforilamino}-propiónico;

isopropil-éster de ácido (S)-2-[[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-1-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-2-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2H-pirimidín-1-il)-2,4-difluoro-3-[((S)-1-isopropoxicarbonil-etilamino)-(naftalén-2-iloxi)-fosforiloxi]-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-2-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-4-metil-3-propioniloxi-tetrahidro-furán-2-ilmetoxi]-(naftalén-1-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[(S)-[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-2-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[(R)-[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-2-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[(R)-[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-1-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[(S)-[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-1-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-{(S)-[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-phenoxi-fosforilamino}-propiónico;

isopropil-éster de ácido (S)-2-{(R)-[(2S,3S,4R,5R)-5-(2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-phenoxi-fosforilamino}-propiónico;

isopropil-éster de ácido (S)-2-[(S)-[(2S,3S,4R,5R)-5-(5-bromo-2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-1-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[(S)-[(2S,3S,4R,5R)-5-(5-bromo-2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-2-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[(R)-[(2S,3S,4R,5R)-5-(5-Bromo-2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-1-iloxi)-fosforilamino]-propiónico;

isopropil-éster de ácido (S)-2-[(R)-[(2S,3S,4R,5R)-5-(5-bromo-2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-2-iloxi)-fosforilamino]-propiónico; y

isopropil-éster de ácido (S)-2-[[(2S,3S,4R,5R)-5-(5-bromo-2,4-dioxo-3,4-dihidro-2*H*-pirimidín-1-il)-2,4-difluoro-3-hidroxi-4-metil-tetrahidro-furán-2-ilmetoxi]-(naftalén-2-iloxi)-fosforilamino]-propiónico.

15. Composición farmacéutica que comprende un compuesto según cualquiera de las reivindicaciones 1 a 14 y un portador terapéuticamente inerte.

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Review

# Ten years of dengue drug discovery: Progress and prospects

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ABSTRACT

To combat neglected diseases, the Novartis Institute of Tropical Diseases (NITD) was founded in 2002 through private–public funding from Novartis and the Singapore Economic Development Board. One of NITD's missions is to develop antivirals for dengue virus (DENV), the most prevalent mosquito-borne viral pathogen. Neither vaccine nor antiviral is currently available for DENV. Here we review the progress in dengue drug discovery made at NITD as well as the major discoveries made by academia and other companies. Four strategies have been pursued to identify inhibitors of DENV through targeting both viral and host proteins: (i) HTS (high-throughput screening) using virus replication assays; (ii) HTS using viral enzyme assays; (iii) structure-based *in silico* docking and rational design; (iv) repurposing hepatitis C virus inhibitors for DENV. Along the developmental process from hit finding to clinical candidate, many inhibitors did not advance beyond the stage of hit-to-lead optimization, due to their poor selectivity, physiochemical or pharmacokinetic properties. Only a few compounds showed efficacy in the AG129 DENV mouse model. Two nucleoside analogs, NITD-008 and Balapiravir, entered preclinical animal safety study and clinic trial, but both were terminated due to toxicity and lack of potency, respectively. Celgosivir, a host alpha-glucosidase inhibitor, is currently under clinical trial; its clinical efficacy remains to be determined. The knowledge accumulated during the past decade has provided a better rationale for ongoing dengue drug discovery. Though challenging, we are optimistic that this continuous, concerted effort will lead to an effective dengue therapy.

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*Abbreviations:* DENV, dengue virus; WNV, West Nile virus; YFV, yellow fever virus; TBEV, tick-borne encephalitis virus; HCV, hepatitis C virus; WEEV, Western equine encephalitis virus; VSV, vesicular stomatitis virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; DAA, direct antiviral agent; PI, protease inhibitors; HTS, high-throughput screening; MTase, methyltransferase; RdRp, RNA-dependent RNA polymerase; NI, nucleoside analog inhibitors; NNI, non-nucleoside inhibitor; PoC, proof-of-concept; TI, therapeutic index; SAR, structure–activity relationship; ER, endoplasmic reticulum; DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; SPA, scintillation proximity assay; FP, fluorescence polarization.

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## 1. An unmet medical need: dengue

Dengue fever (DF), also known as 'breakbone fever', is caused by dengue virus (DENV). The four serotypes of DENV constitute the most significant mosquito-borne viral pathogen. The virus is transmitted by infected *Aedes* (*A.*) mosquitoes, primarily *A. aegypti*. Dengue is endemic in over 100 countries across the globe, and on every continent except Antarctica. A recent study estimated that 390 million people are infected with DENV each year, with 96 million infections exhibiting disease symptoms each year (Bhatt et al., 2013). Upon DENV infection, some individuals develop mild disease with flu-like symptoms, whereas a few individuals develop the severe disease – dengue hemorrhagic fever and shock syndrome (DHF/DSS). The acute phase of the illness, characterized by fever and myalgias, lasts 1–2 weeks. Convalescence is accompanied by asthenia, and full recovery often takes several weeks. Dengue is a major cause of morbidity and hospitalization in many tropical and subtropical countries. The greatest documented burden of dengue occurs in Asia and Latin America, but the disease has also emerged in previously unaffected areas. It continues to spread, fueled by changing climates, increasing urbanization, and global travel. The cost of illness to society is considerable, from lost wages and diminished productivity, to costs related to care-giving and direct medical expenses. The cost of dengue in the Western Hemisphere alone is estimated at $2.1 billion per year (Beatty et al., 2011).

Public–private partnership is an excellent model to fight neglected diseases. The Novartis Institute for Tropical Diseases (NITD) was founded in 2002 through funding from Novartis and the Singapore Economic Development Board. One of NITD's missions is to develop novel therapeutics for dengue; such therapeutics will be provided to poor patients in developing countries at a cost price. To seek feedback on potential treatment strategies, a symposium supported by the Novartis Foundation, was held at NITD in 2005, with participation of leading flavivirologists from around the world (Novartis Foundation Symposium 277, 2006). NITD remains the only institution that has a dedicated mission of dengue antiviral development. In the past decade, NITD together with academia and other companies have made significant inroads towards the development of dengue antiviral. The goal of this review is to summarize major progress on this topic.

## 2. Feasibility of dengue antiviral therapy

One key concern surrounding the utility of antiviral therapy for dengue is the rapid decline in viremia during the natural course of infection. Studies of dengue patients indicate that viremia is reduced up to 10-fold within 24 h and up to 100-fold within 48 h (Libraty et al., 2002a,b; Nguyen et al., 2013). Thus, a DENV antiviral must be fast-acting and will be more effective when used in tandem with rapid diagnostics of early DENV infection. On this note, researchers and clinicians have been searching for prognostic biomarkers for severe dengue diseases. Nevertheless, there is sufficient precedent to suggest that antivirals will work for dengue.

Direct antiviral agents (DAA) have been successfully used to treat chronic viral diseases such as HIV-1, HCV, Hepatitis B virus (HBV), as well as acute viral illness like influenza. Thus, the treatment strategy for DENV would be akin to that for influenza, with a higher likelihood of success when patients are dosed as early as possible, ideally within 40 h of fever onset. In urban settings, this would not be a problem, as clinical assessment, coupled with rapid diagnostic kits (e.g., viral NS1 protein test) can be utilized. In rural areas, however, where laboratory services are not readily available, reliance on experienced medical personnel to identify individuals with traits of DENV infection is crucial. Eventually, the feasibility of dengue antiviral therapy can only be unequivocally addressed in patients using a clinical candidate. When an antiviral becomes available, dengue patients will readily seek treatment; such patient behavior would greatly facilitate early treatment. In addition, an antiviral could potentially be used prophylactically in communities with dengue outbreaks.

These considerations mandate that the optimal and minimally acceptable characteristics of dengue drugs (target product profiles) must encompass a good safety profile, with the eventual intent to treat children, since they make up a significant number of the patient population in developing countries. Other optimal characteristics include rapid resolution of symptoms and reducing risks of severity, comparable inhibition of all four DENV serotypes, oral administration and a long shelf-life to facilitate drug distribution in rural settings. Ideally, an effective dengue drug would be given to patients orally once a day, and for less than a week.

## 3. Rationale for a direct antiviral agent for dengue

Distinct differences (>10-fold) in plasma viremia between severe (DHF/DSS) and non-severe (DF) patients have been reported in the research literature (Libraty et al., 2002a,b). This suggests that reducing viral levels by antiviral therapy during the early phase of infection could prevent or lessen the chances of patients progressing to DHF/DSS. Both Wang et al. (2003, 2006) and Guilarde et al. (2008) observed the presence of DENV during post-defervescence in patients with severe dengue. These findings imply that the clinical manifestations of severe dengue may, in part, be virus-driven, and support the hypothesis that antivirals given at later stages of illness will still be beneficial.

Based on the clinical results, the current rationale for a dengue antiviral is to rapidly reduce viremia by >10-fold through DAA treatment during the early phase of DENV infection; such reduction of viremia would be expected to translate into clinical benefits and prevent the development of DHF/DSS. However, it remains to be established whether the 10-fold reduction of viremia should be the only criterion to determine the minimal efficacious dose during preclinical development or as the key parameter for PoC (proof-of-concept) in a clinical trial. Ideally, prevention of the infected patients from development into severe diseases (DHF/DSS) should be used as the ultimate PoC criteria. Practically, since <1% of dengue patients develop DHF/DSS (St John et al., 2013), testing the effect of a compound on the prevention of DHF/DSS would require more than several thousand patients in order to draw a statistically sound conclusion. Therefore, it is critical to identify prognostic biomarkers that can predict which patients would develop severe disease. Such prognostic biomarkers would greatly help healthcare workers to stratify dengue patients and to facilitate antiviral trials.

To address this goal, the Early Dengue Infection and Outcome Study (EDEN) was undertaken in 2005 by NITD and researchers from 7 Singapore agencies (Low et al., 2006). This multi-center longitudinal study characterized the epidemiological, clinical, viral and host-specific features of adult dengue disease. Increased expression of IP-10 and I-TAC in the peripheral blood of dengue patients at the early onset of fever was detected and may be useful as early biomarkers (Fink et al., 2007). The study also enabled the generation of decision algorithms using simple clinical and hematological parameters that predicted diagnosis and prognosis of dengue which could be useful in disease management and surveillance (Tanner et al., 2008; Schreiber et al., 2009).

## 4. Viral genome and replication

DENV belongs to the family *Flaviviridae*, which includes three genera, *Flavivirus*, *Pestivirus*, and *Hepacivirus*. DENV is a member of the genus *Flavivirus*, which includes other disease-causing viruses such as West Nile virus (WNV), yellow fever virus (YFV), tick-borne encephalitis virus (TBEV), and Murray Valley encephalitis virus (MVEV). DENV is spherical in shape, with a diameter of 50 nm. The structures of mature and immature DENV particles have been solved by cryo-electron microscopy (Mukhopadhyay et al., 2005). The virus has a single-stranded, plus-sense RNA genome approximately 11,000 nucleotides in length. The genomic RNA encodes 10 proteins (Fig. 1): the structural proteins capsid (C), envelope (E), and membrane (M) and the nonstructural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5. Structural proteins form the virion: the C protein encapsulates the viral genomic RNA to form the nucleocapsid, and the nucleocapsid is enveloped by a lipid bilayer, in which viral prM and E proteins are embedded (Kuhn et al., 2002). The structures of the E (Modis et al., 2003; Rey et al., 1995), prM (Li et al., 2008), and C (Ma et al., 2004a) proteins have been solved by X-ray crystallography or NMR (reviewed in (Perera and Kuhn, 2008)).

Flavivirus nonstructural proteins are essential for virus replication, virion assembly, and evasion of host immune response (Bollati et al., 2010; Kummerer and Rice, 2002; Lindenbach et al., 2007; Liu et al., 2003; Munoz-Jordan et al., 2003). The nonstructural proteins are primarily localized in the cytoplasm to form replication complexes which are involved in viral RNA synthesis (Lindenbach et al., 2007). The best characterized DENV nonstructural proteins are NS3 and NS5, which are multifunctional proteins containing several enzymatic activities. The N-terminal domain of NS3 is a serine protease (with NS2B as a cofactor) and its C-terminal domain encodes RNA triphosphatase and helicase activities (Lescar et al., 2008; Luo et al., 2008a,b; Noble et al., 2012; Noble and Shi, 2012; Xu et al., 2005). NS5 contains a methyltransferase (MTase) domain at its N terminus and an RNA-dependent RNA polymerase (RdRp) at its C terminus (Ackermann and Padmanabhan, 2001; Dong et al., 2008; Egloff et al., 2002; Ray et al., 2006; Yap et al., 2007). Inhibition of viral enzymes has been proven to be the most successful antiviral approach, as demonstrated by the marketed drugs for HIV, HBV, and HCV. Crystal structures of all DENV enzymes have been solved in the past decade (Noble et al., 2010, 2012); such structural information forms a solid foundation for modern drug discovery.

Other nonstructural proteins (NS2A, NS2B, NS4A, and NS4B) are transmembrane proteins that have no identified enzymatic activities, but are essential for formation of the active viral replication complex (Lindenbach et al., 2007; Miller et al., 2007, 2006). Inhibitors targeting these non-structural proteins could be identified using virus replication-based assays. Indeed, using DENV infection assay or replicon assays, several groups have independently identified compounds that inhibit DENV replication by targeting NS4B (see later sections). Nonstructural proteins with no enzymatic activities could also be targeted for antiviral development, as demonstrated by the success of HCV NS5A inhibitor currently in clinical trial (Gao et al., 2010).

## 5. Approaches to find dengue virus inhibitors

Over the past ten years, NITD has taken both target-based and cell-based approaches to identify DENV-specific inhibitors. For the target-based approach, we systematically developed *in vitro* biochemical assays for DENV protease (Li et al., 2005), helicase (Krawczyk et al., 2010), methyltransferase (Chung et al., 2010;

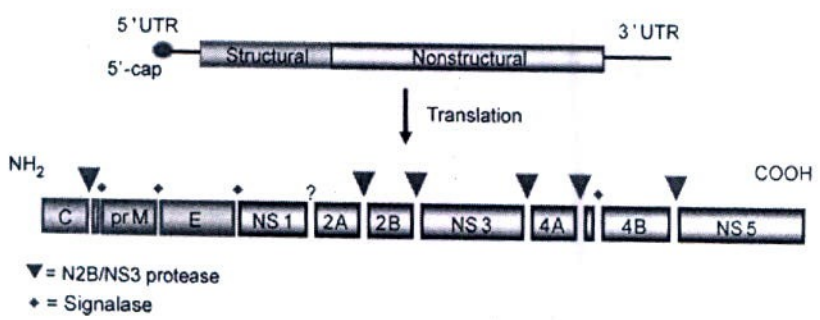



**Fig. 1.** Schematic diagram of the DENV genome organization and polyprotein processing. At the top is the viral genome, with the 5'-cap, 5' UTR, the structural and nonstructural protein coding regions, and 3' UTR. The bottom figure depicts the genome-translated polyprotein that is processed by the viral NS2B/NS3 protease, host signalases or unknown proteases.

Lim et al., 2008), and polymerase (Niyomrattanakit et al., 2010a). Screening campaigns have been performed against each of these targets. Unfortunately, though much effort was made to follow up on hits generated from these screens, none yielded specific inhibitors with good lead-like properties. In addition to the enzyme activity-based HTS, we also applied structure-based *in silico* docking (e.g., E protein inhibitor (Wang et al., 2009)) and rational design (e.g., methyltransferase inhibitor (Lim et al., 2011)). As discussed in the following sections, each target-based project presents its unique set of advantages and challenges.

For the cell-based approach, we performed HTS using three distinct viral replication assays: a DENV infection assay (Noble et al., 2010); a replicon assay (Ng et al., 2007); and a virus-like particle infection assay (Qing et al., 2010). Among the three HTS assays, the virus-like particle infection HTS yielded the least productive hits. Two points are noteworthy for the cell-based approach. First, "hits" identified from these cell-based HTS could potentially inhibit either host or viral targets. The number of host factors required for a productive viral infection cycle is significantly more than 10 viral proteins of DENV. Consequently, the probability of identifying inhibitors of host proteins in cell-based assays is anticipated to be much greater than that for viral proteins. It is therefore critical to biologically profile the "hits" to understand their antiviral mechanisms and targets before launching into chemical derivations of the hit. Secondly, target deconvolution can be challenging. The HTS hits could be tested in the various DENV enzyme assays to screen for viral targets; alternatively, the viral target could be identified through selection for resistant virus (Xie et al., 2011); in addition, a compound-mediated pulldown method could be used to identify host targets (Bonavia et al., 2011; Wang et al., 2011a).

In general, hits optimized in the target-based screening approaches are validated in the DENV cell-based assays for on-target antiviral activity. Depending on its physicochemical property, a compound class from target-based approaches may need to be further improved for cellular permeability. On the other hand, hits from cell-based screening efforts usually do not have this issue. Compounds that show anti-DENV activity in cells are further assessed for cytotoxicity and selectivity against other related viruses (such as WNV, JEV, YFV or HCV) or unrelated viruses (e.g. alphaviruses). In the final stages, lead compounds are selected for *in vitro* and *in vivo* pharmacokinetic profiling including plasma stability (half-life), exposure and oral bioavailability, before being tested in dengue mouse efficacy models (Schul et al., 2007; Yin et al., 2009b) and two-week preclinical toxicity studies in animals (usually in rats and dogs). On this note, selection of starting compounds that have lower molecular weight (MW <300 Da), lower lipophilicity, and fewer hydrogen bonds is more likely to result in optimized leads with acceptable drug-like properties (reviewed in Verheij, 2006). These chemical entities with molecular properties that adhere to Lipinski's "rule of five" (<5 hydrogen bond donors, <10 hydrogen bond acceptors, LogP <5, MW <500 Da) often have lower attrition rates during preclinical and clincal developments (Lipinski, 2004).

The following sections review key dengue drug discovery progress made at the NITD, as well as major discoveries made by academia and other companies in the past ten years.

## 6. Entry inhibitors

Inhibiting viral entry is a proven antiviral approach, as exemplified by two marketed drugs for HIV-1treatment. Maraviroc interacts with HIV co-receptor CCR5, inhibiting viral attachment to cells and entry (Pugach et al., 2008). Enfuvirtide binds to viral gp41, blocking viral and host cell membrane fusion (Lalezari et al., 2003). In addition, Myrcludex-B, a synthetic lipopeptide derived from the preS1 domain of the hepatitis B virus (HBV) envelope protein, which targets the hepatocytes and blocks *de novo* HBV infection, is currently undergoing phase Ib-IIa trials (Volz et al., 2013). Of the three inhibitors, the latter two are of peptide origin and must be administered by subcutaneous injection.

X-ray crystallography and cryo-electron microscopy studies have revealed that DENV consists of 180 molecules of E and M proteins arranged on a host-derived lipid membrane (Mukhopadhyay et al., 2005). Each monomer of the E ectodomain consists of three domains: I, II, and III. Two features of DENV E protein and DENV membrane fusion could be used for design of entry inhibitors (Fig. 2). First, between domain I and domain II is a hydrophobic pocket that was found to interact with detergent *β*-N-octyl-glucoside (Modis et al., 2003). Inhibitors that occupy this pocket could sterically block the conformational changes between domains I and II; such conformational change is essential for virus–host membrane fusion. Second, the C-terminal portion of the E protein consists of a stem region, followed by a membrane anchor. The stem region is highly conserved among flaviviruses; during fusion, the stem region needs to fold back onto the trimer of E ectodomain. Similar to HIV Enfuvirtide, a peptide representing the DENV E stem could competitively block membrane fusion (see examples below).

Four main approaches have been taken to search for entry inhibitors of DENV:

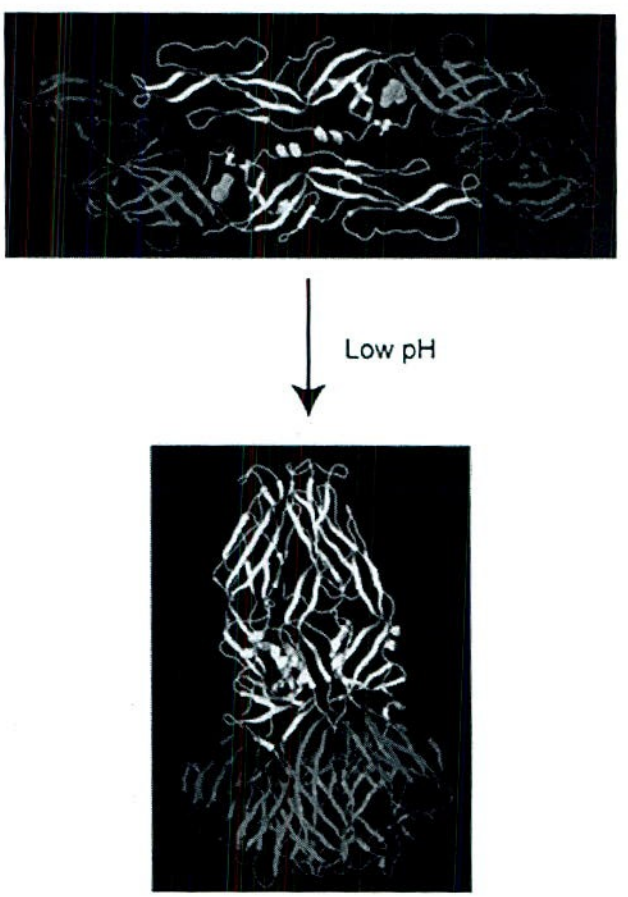



**Fig. 2.** The DENV E dimer and trimer. The red, yellow and blue parts of each subunit correspond to domains I, II and III of the DENV E protein, respectively. The *n*-octyl-β-D-glucoside (β-OG) is displayed as green spheres. Upon acidification, the pre-fusion E dimer undergoes a conformational change to the fusion-active E trimer (Modis et al., 2003, 2004).

(i) *In silico* docking to identify compounds that bind to the hydrophobic pocket between domains I and II;
(ii) compound library screening, using an assay that captures a fusion intermediate between a stem-less E trimer and an E stem peptide;
(iii) design of peptides that block the fold-back interaction between the E stem and E trimer during fusion;
(iv) diverse library screens, using a DENV infection assay.

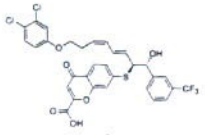
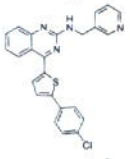
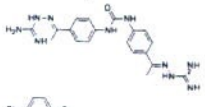
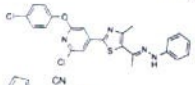
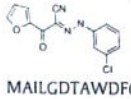
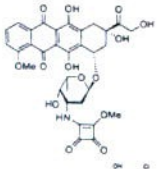
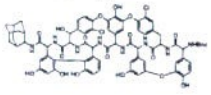
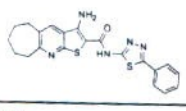
These approaches have produced some promising results.

### 6.1. Targeting a hydrophobic pocket of the E protein

A virtual screen has been performed to identify small molecules that bind to the hydrophobic pocket for β-N-octyl-glucoside in the DENV E protein (Fig. 2; (Alen and Schols, 2012)). Two classes of compounds were identified from the Novartis compound library. The first class of compounds, represented by NITD-448 (Table 1), inhibited E protein-mediated membrane fusion ($IC_{50}$ 6.8 μM) as well as DENV-2 infection ($EC_{50}$ 9.8 μM) in cell culture (Poh et al., 2009). Due to the large molecular weight of this compound, poor selectivity, and pharmacokinetic properties, this compound series was not further pursued. The second class of compounds (e.g., compound 6; Table 1) contained a quinazoline core, and inhibited various laboratory and clinical isolates of DENV with micromolar to nanomolar $EC_{50}$ (Wang et al., 2009). After significant chemistry efforts to improve the pharmacokinetic properties of this scaffold, its low potency and high plasma protein-binding activity due to lipophilicity prevented further development of the compound.

*In silico* docking with the NCI library also identified the highly basic compound P02 (Table 1), which inhibited YFV production at micromolar concentrations (Zhou et al., 2008). This compound was confirmed by NMR to bind DENV virions; in addition, it competed with *N*-octyl-β-D-glucoside for binding to the E protein. Kampmann et al. (2009) also performed a virtual screen using the Maybridge database. One compound, A5 (Table 1), was found to have low micromolar activity against DENV, WNV, and YFV. The compound prevented syncytia formation in cells expressing DENV envelope. However, no follow-up studies have been reported for these compounds.

**Table 1**
Inhibitors of DENV structural proteins (see details in text).

| Compound | Structure | Mode of action | Cellular activity ($EC_{50}/CC_{50}$) μM | AG129 dengue mouse activity | References |
|---|---|---|---|---|---|
| Entry inhibitors | | | | | |
| NITD-448 |  | E protein βOG pocket | 9.8/48.7 | ND | Poh et al. (2009) |
| Compound 6 |  | E protein βOG pocket | 0.119/>20 | ND | Wang et al. (2009) |
| P02 |  | E protein βOG pocket | 13[a]/371 | ND | Zhou et al. (2008) |
| A5 |  | E protein βOG pocket | 1.2/>100 | ND | Kampmann et al. (2009) |
| 1662G07 |  | E stem and E trimer interaction | 16.9[b]/>100 | ND | Schmidt et al. (2012) |
| Peptide DN59(stem peptide) | MAILGDTAWDFGSLGGVFTSIGKALHQVFGAIY | Bind to E trimer to block stem binding | 10/>30 | ND | Hrobowski et al. (2005) |
| Peptide 1OAN1 (β sheet connecting E domains I and II) | FWFTLIKTQAKQPARYRRFC | Interact with E to block virus attachment | 7/>50 | ND | Costin et al. (2010), Nicholson et al. (2011) |
| Antibiotic SA-17 |  | Entry, possibly through E βOG pocket | 0.52/43 | ND | Kaptein et al. (2010) |
| Antibiotic LCTA-949 |  | Entry | 6.9/>25 | ND | De Burghgraeve et al. (2012) |
| Capsid inhibitor | | | | | |
| ST-148 |  | C protein | 0.016/>100 | Efficacious | Byrd et al. (2013a) |

[a] Activity against YFV is presented.
[b] $EC_{90}$ is presented. ND, not determined.

### 6.2. Targeting the interaction between E stem and E trimer

Since the stem region of E needs to interact with E trimer during DENV fusion, Schmidt et al. (2012) developed a fluorescence polarization (FP) assay that detects the binding of an E stem-derived peptide to a stem-less E trimer. Using this assay, they uncovered a series of compounds (e.g., 1662G07; Table 1) that inhibit DENV-2. Structure–activity relationship (SAR) studies led to analogs with sub-micromolar $IC_{90}$s against DENV-2, some activity against DENV-4, but weak activity against DENV-1 and -3. The antiviral spectrum of the compounds needs to be broadened to inhibit all four serotypes.

Besides the small-molecule approach described above, peptides could also be designed to inhibit E-mediated viral entry. Several groups have demonstrated that short peptides derived from the DENV E protein can inhibit DENV in cell culture (Costin et al., 2010; Hrobowski et al., 2005; Liao and Kielian, 2005; Lok et al., 2012). These peptides could also inhibit antibody-enhanced DENV infection (Nicholson et al., 2011). Peptide DN59 (Table 1), representing the pre-membrane stem region (amino acids 412–444) of DENV-2 E, bound to E trimer to inhibit fusion. Such stem peptides inhibited all four DENV serotypes (Schmidt et al., 2010b). Remarkably, DN59 induced holes on virion to eject nucleocapsid, giving rise to empty virions; the mechanism of peptide-induced nucleocapsid ejection remains to be determined (Lok et al., 2012).

Peptide 1OAN1 (Table 1) is a computationally optimized mimic of the first beta sheet strand that connects domains I and II of E protein; the peptide interferes with virus binding to cell through direct interaction with E protein (Nicholson et al., 2011). Both DN59 and 1OAN1 interact with E protein with dissociation constants of about $10^{-7}$ M (Costin et al., 2010; Schmidt et al., 2010a). Further optimization is needed to improve the potency of these peptide inhibitors. The drawbacks of peptidic inhibitors are the need for intravenous administration and its low-shelf life which could limit their use in clinics. Given that dengue is prevalent mostly in developing countries, it will be challenging to make this class of inhibitors accessible to poor patients living in rural areas.

### 6.3. Entry inhibitors with undefined mechanisms

Using a DENV infection assay, Neyts and colleagues identified two classes of antibiotics that inhibit DENV with micromolar activities in cell culture. The two classes of antibiotics inhibitors are SA-17 (a derivative of doxorubicin; Table 1) and LCTA-949 (a glycon analog of the antibiotic teicoplanin; Table 1) (De Burghgraeve et al., 2012; Kaptein et al., 2010). Time-of-addition experiments suggest that both compounds inhibit an early step of the viral infection cycle; neither compound inhibited a DENV replicon, which lacks viral entry. These results indicate that these compounds act as entry inhibitors. In addition, LCTA-949 was shown to directly bind to DENV particles and to prevent infection of DENV opsonized with antibodies. *In silico* docking suggests that SA-17 fits into the β-N-octyl-glucoside pocket of the E protein.

Besides the small-molecule approach, monoclonal antibodies have been actively pursued for therapeutic purposes. The main challenge of this approach is to design an antibody that can potently neutralize all four serotypes of DENV. Since this review focuses on small-molecule inhibitors, we will not discuss therapeutic antibodies. Readers are encouraged to refer to an excellent review on this topic (Diamond et al., 2012).

## 7. Capsid inhibitors

From a DENV infection-based HTS of approximate 200,000 compounds, Byrd et al. (2013a) identified an inhibitor, ST-148 (Table 1), which targets the DENV C protein. This compound inhibited DENV-2 in a viral titer reduction assay with an $EC_{50}$ of 0.016 μM and an $EC_{90}$ of 0.125 μM. It displayed weaker activities against other serotypes, with $EC_{50}$s ranging from 2.832, 0.512, and 1.150 μM for DENV-1, -3, and -4, respectively. ST-148 resistant DENV-2 harbored a single amino acid change of S34L in C protein. Infectious virus with the engineered mutation showed a 550-fold reduction of susceptibility to ST-148. Binding of ST-148 to DENV C protein was also performed; surprisingly, the compound bound equally well to the wild-type and mutant C proteins, raising the question of the mechanism-of-inhibition of this compound. Ser34 is located in the α1–α1′ helices of the dimeric C structure. The authors proposed that ST-148 might prevent C interaction with membranes.

Oral and intraperitoneal administrations of ST-148 showed good exposure when dosed at 50 mg/kg/day in the AG129 mice with peak plasma concentrations at 468-, and 7750-fold above the *in vitro* $EC_{50}$. Similarly, after an intravenous dose of 20 mg/kg/day, mice plasma levels of ST-148 were 6093-fold above the *in vitro* $EC_{50}$. On average, BID treatment with ST-148 reduced peak plasma viremia by 52-fold and reduced viral load in the spleen and liver by 3- and 20-fold, respectively. The pharmacokinetic data indicate that ST-148 has limited oral bioavailability and fairly rapid clearance but good systemic availability following i.p. administration. Overall, the results warrant further preclinical development of ST-148.

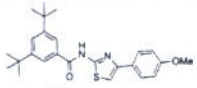
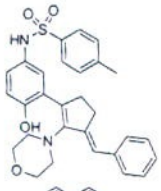
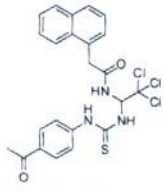
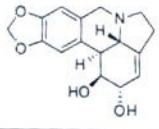
## 8. NS4B inhibitors

Using an HTS with a cell line harboring a luciferase replicon of DENV-2, we identified a DENV NS4B inhibitor, NITD-618 (Table 2; (Xie et al., 2011)), from a compound library of 1.8 million small molecules. NITD-618 was active against all four serotypes, with $EC_{50}$s ranging from 1.0 to 4.1 μM, but inactive against other RNA viruses ($EC_{50}$ >40 μM), including the two closely related flaviviruses, WNV and YFV, two plus-strand RNA alphaviruses (chikungunya virus and Western equine encephalitis virus [WEEV]), and a negative-strand RNA rhabdovirus (vesicular stomatitis virus [VSV]). These results demonstrate that NITD-618 selectively inhibits DENV. Mapping of the NITD-618-resistant replicon revealed amino acid changes within the NS4B protein (P104L and A119T); when engineered into the wild-type replicon or DENV, these mutations conferred resistance to NITD-618. This compound could interrupt the NS3–NS4B complex formation, as residue P104L was previously shown to be important for the NS3–NS4B interaction (Umareddy et al., 2006). Unfortunately, the high lipophilicity of NITD-618 resulted in poor pharmacokinetic properties which hindered testing its activity against DENV in AG129 mice. Medicinal chemistry attempts to reduce its lipophilicity resulted in a loss of activity or a reduction in antiviral selectivity against DENV.

Other groups working on related flaviviruses have also found inhibitors that target the NS4B protein. An HTS using pseudo-infectious YFV particles, which express *Renilla* luciferase in a replication-dependent manner, identified two classes of inhibitors targeting the NS4B protein (Patkar et al., 2009). Virus resistant to compounds CCG-3394 and CCG-4088 (Table 2) harbored a K128R mutation in the cytoplasmic loop of NS4B.

Lycorine (Table 2) was reported to reduce viral titers of WNV, DENV, and YFV mainly through suppression of viral RNA replication (Zou et al., 2008). An amino acid change V9M in viral 2 K peptide conferred WNV resistance to lycorine through enhancing viral RNA replication. Initial chemistry synthesis demonstrated that modifications of the two hydroxyl groups of lycorine can increase the compound's potency, while reducing its cytotoxicity. In HCV, an NS4B inhibitor, Clemizole, was also identified from an HTS

**Table 2**
Inhibitors of DENV NS4B (see details in text).

| Compound | Structure | Mode of action | Cellular activity ($EC_{50}/CC_{50}$) μM | AG129 dengue mouse activity | Reference |
|---|---|---|---|---|---|
| NITD-618 |  | NS4B | 1.0/>40 | ND | Xie et al. (2011) |
| CCG-3394 |  | NS4B | 1.48/31 | ND | Patkar et al. (2009) |
| CCG-4088 |  | NS4B | 0.4/13 | ND | Patkar et al. (2009) |
| Lycorine |  | 2 K peptide | 0.23/24 | ND | Zou et al. (2008) |

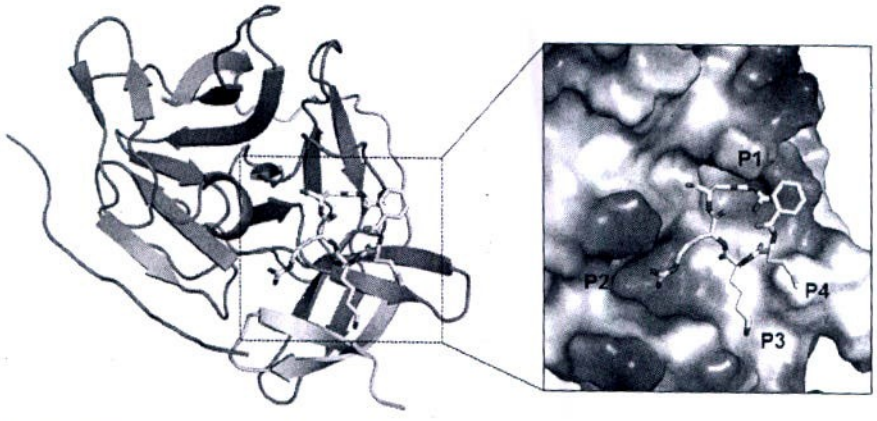



**Fig. 3.** Structure of the DENV NS2B/3 protease, highlighting the S2, S3 pockets, with the bound nKRR-H inhibitor (Noble et al., 2012). The structure is shown in cartoon representation, with NS3 in pink and NS2B in green. The wrapped NS2B beta-hairpin is indicated. Bz-nKRR-H is shown in stick representation.

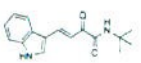
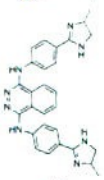
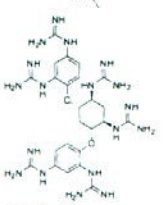
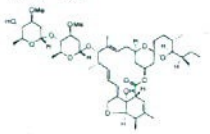
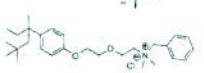
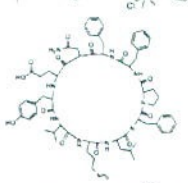
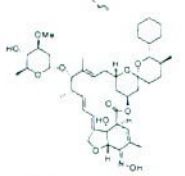
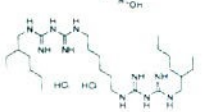
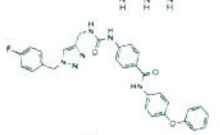
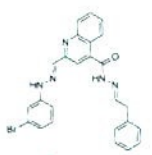
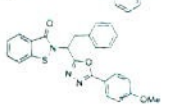
campaign; Clemizole is currently in Phase I clinical trial (Einav et al., 2010).

## 9. Protease inhibitors

Viral proteases are proven antiviral targets, as evidenced by the clinical availability of ten HIV-1 protease inhibitors (PIs) (De Clercq, 2009) and the two HCV PIs (Wyles, 2013). Thus, it is plausible that a protease inhibitor for DENV would be efficacious in the clinic. However, experience with both HIV-1 and HCV indicates that there are certain drawbacks associated with PIs, and these are important considerations in developing a DENV PI. For the treatment of both HIV-1 and HCV infection, the rapid emergence of PI drug-resistant viral strains was observed. This limits their utility to combination therapy with other DAAs (or additionally, in the case of HCV, with PegIFN/ribavirin), as well their prospects for long-term therapy (Wensing et al., 2010). Furthermore, while the majority of HIV-1 PIs are active across different viral strains, HCV PIs (telaprevir and boceprevir) are active only against genotype 1 (as drug design was based on the protease structure of HCV genotype 1), and are less effective in genotype 1a than 1b (Pockros, 2011). Sequence dissimilarity in the NS3 PI binding sites, occurring as natural polymorphisms across the different HCV genotypes, explains why non-genotype-1 HCV strains are resistant to both PIs (Halfon and Locarnini, 2011). More worrisome, naturally occurring mutations in NS3 have been detected in a significant number of HCV genotype 1 strains in treatment-naïve patients; these mutations result in resistance to the two PIs (Halfon and Locarnini, 2011).

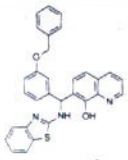
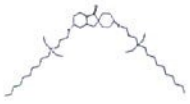
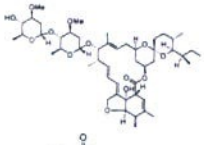
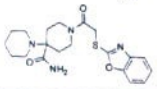
The DENV protease bears strong functional similarity to its HCV counterpart. Like the latter, DENV NS3 protease activity is dependent on association with a hydrophilic region of 40 amino acids of NS2B. Unlike HCV, NS2B functions as a cofactor that actively participates in the formation of the S2 and S3 sub-pockets in the protease active site (Fig. 3; (Erbel et al., 2006; Noble et al., 2012)). Proteases from the four serotypes of DENV share very similar substrate specificity (Li et al., 2005), as well as higher sequence

**Table 3**
Inhibitors of DENV NS3 protease and helicase (see details in text).

| Compound | Structure | Mode of action | Cellular activity ($EC_{50}/CC_{50}$) μM | AG129 dengue mouse activity | References |
|---|---|---|---|---|---|
| *Protease inhibitors* | | | | | |
| Retro tripeptides | R-Arg-Lys-Nle-$NH_2$ | Mixed inhibition | ND | ND | Nitsche et al. (2012) |
| Cyclic peptide 9 | Cys-Ala-Gly-Lys-Arg-Lys-Ser-Gly | Protease | ND | ND | Xu et al. (2012) |
| Tetrapeptide | Bz-Nle-Lys-Arg-Arg-$B(OH)_2$ | Competitive inhibition | ND | ND | Yin et al. (2006) |
| Compound 32 |  | Protease | <10/>100 | ND | Steuer et al. (2011) |
| Compound 1 |  | Competitive inhibition | ND | ND | Bodenreider et al. (2009) |
| 166347 |  | Competitive inhibition | ND | ND | Cregar-Hernandez et al. (2011) |
| ARDP0006 |  | Competitive inhibition | 4.2/69 | ND | Tomlinson et al. 2009a,b |
| Ivermectin |  | Mixed inhibition | ND | ND | Tomlinson and Watowich (2012) |
| Benzethonium chloride |  | Mixed inhibition | ND | ND | Tomlinson and Watowich (2012) |
| Tyrothricin |  | Competitive inhibition | ND | ND | Tomlinson and Watowich (2012) |
| Selamectin |  | Mixed inhibition | ND | ND | Tomlinson and Watowich (2012) |
| Alexidine hydrochloride |  | Mixed inhibition | ND | ND | Tomlinson and Watowich (2012) |
| Aminobenzamide |  | Protease | ND | ND | Aravapalli et al. (2012) |
| Compound 23i |  | Protease | 24.7/>100 | ND | Deng et al. (2012) |
| Compound 7n |  | Protease | ND | ND | Lai et al. (2013a) |

(continued on next page)

**Table 3** (*continued*)

| Compound | Structure | Mode of action | Cellular activity ($EC_{50}/CC_{50}$) μM | AG129 dengue mouse activity | References |
|---|---|---|---|---|---|
| Compound 1 |  | Protease | ND | ND | Lai et al. (2013b) |
| BP2109 |  | Protease | 0.17/29.28 | ND | Yang et al. (2011) |
| *Helicase inhibitors* | | | | | |
| Ivermectin |  | Helicase | 0.7/3.8 | ND | Mastrangelo et al. (2012) |
| ST-610 |  | Helicase | 0.272/>100 | | Byrd et al. (2013b) |

similarity, compared to HCV proteases. In 1,500 HCV NS3-protease sequences analyzed from treatment-naive patient samples, only 47% of amino acids were found to be conserved (Cento et al., 2012). Along the same line, sequence similarity across DENV serotypes for NS3 is between 63% to 74%, suggesting that the genetic barrier to develop resistance could also be low for DENV PI. Therefore, a dengue PI would have to be administered in combination with other DAAs.

With the exception of tipranavir, most HIV-1 PIs and the two HCV PIs are substrate mimetics. This approach has been similarly taken to develop DENV PI, but to date, no compounds have advanced to the preclinical stage. The challenges with the peptidiomimetic strategy for DENV protease are the shallow active site (like in HCV) and the need to replace the two basic (mostly Arg) P1 and P2 residues (Fig. 3). Most of the reported DENV peptidic inhibitors possessed a reactive warhead and/or at least one basic residue. As such, they exhibit inhibitory activities in biochemical protease assays (retro tripeptide, cyclic peptide 9, tetrapeptide, and compound 32; Table 3), but not in cell-based viral infection assays (except for compound 32 (Steuer et al., 2011); Table 3) due to their poor permeability and stability (Nitsche et al., 2012; Xu et al., 2012; Yin et al., 2006). Cyclizing the peptides improves their stability and cell permeability, and could be a way forward for DENV PI (Xu et al., 2012).

The most potent DENV peptidic inhibitor is a tetra-peptide with a boronic acid, which is highly unsuitable for oral dosing and is non-DENV specific (Yin et al., 2006). This is because DENV protease shares substrate specificity with the host serine protease, furin (bearing the cleavage site RXRR), and to a lesser extent, other serine proteases like trypsin, thrombin, or elastase, which recognize Arg in the P1 position. We observed that the peptidic inhibitor, Bz-Nle-Lys-Arg-Arg-H was highly potent against these human enzymes when tested *in vitro* (unpublished). Cross inhibition against human enzymes is a safety concern in the design of DENV PIs. Therefore, a larger selectivity panel of host serine proteases would be needed to ascertain DENV specificity.

The second approach to identify DENV PIs relies on diverse library screening. Several campaigns have been carried out with the single-chain linked NS2B/3 protein construct (Leung et al., 2001). A number of inhibitors have been reported. For one particular class of compound (compound 1; Table 3) identified at NITD, the ensuing hit-to-lead activities generated both specific and nonspecific analogs that could be differentiated by biophysical assays (Bodenreider et al., 2009). NMR showed that the closed conformation of the protease was stabilized upon binding of compound 1 and its specific analogs but not of nonspecific compounds. This experience illustrates the pitfalls in relying solely on $IC_{50}$ values to drive the SAR in hit-to-lead optimization. However, these compounds did not show activity in cell-based assays, probably due to poor peameability caused by the presence of positively charged substituents in this scaffold.

Several other compound series have been identified elsewhere. However, many of the lead compounds are weakly active (single-digit micromolar inhibition), have not been shown to directly bind to the DENV protease *in vitro*, and do not show inhibition in cellular assays, prompting speculation that some of these hits may be nonspecific. If co-crystal structures of these compounds bound to the DENV protease can be generated, this will certainly guide lead optimization. A guanidinylated and therefore cationic compound (166347; Table 3) was identified as a competitive inhibitor, suggesting that it bound to the active site of the DENV protease (Cregar-Hernandez et al., 2011). Separately, Tomlinson et al. 2009a,b and Tomlinson and Watowich (2011, 2012) identified several classes of compounds (ARDP0006, Ivermectin, benzethonium chloride, tyrothricin, selamectin, and alexidine hydrochloride; Table 3) that inhibited DENV-2 protease, some of which also inhibited the WNV protease, but were not active against trypsin. Lai and colleagues showed that the protease hits (Aminobenzamide, 23i, 7n, and 1; Table 3) identified from a WNV protease screen could also inhibit the DENV protease; furthermore, these hits could be docked onto the substrate S1–S4 binding site on the protease structure (Aravapalli et al., 2012; Lai et al., 2013a,b).

A rational approach using *in silico* docking identified inhibitors of the DENV protease (compound 23i; Table 3; (Deng et al., 2012)). Although the compounds showed activity in a biochemical protease assay, none of them had TI (therapeutic index = $CC_{50}/EC_{50}$) of $\geqslant 10$. Yang and colleagues identified a hit (BP2109; Table 3) through a DENV protease HTS (Yang et al., 2011). BP2109 exhibited low micromolar inhibition *in vitro*, but submicromolar activity in a replicon assay. Importantly, a BP2109-resistant DENV replicon revealed two amino acid mutations (R55K and E80K) in NS2B, which when engineered into DENV resulted in the loss of sensitivity to the compound. The R55K and E80K mutations were mapped to the soluble region of NS2B that form part of the active protease.

BP2109 (Table 3) contains two positive charges, but is likely cell permeable due to the formation of micelle and cellular internalization through endocytosis. Although this compound is not a good starting point for lead optimization, it demonstrates that the DENV protease is a druggable antiviral target.

## 10. Helicase inhibitors

The C-terminal domain of the NS3 protein is a non-processive NTPase/helicase (comprising amino acids 180–618). The flavivirus helicase belongs to the superfamily of "SF2" helicases (Lescar et al., 2008). The helicase/NTPase activities and crystal structures have been reported for DENV (Fig. 4), YFV, and JEV (Lescar et al., 2008; Wu et al., 2005; Xu et al., 2005; Yamashita et al., 2008). The ATPase active site is housed between subdomains 1 and 2 with the substrate, ATP, primarily held through its triphosphate moiety via contacts through a divalent metal ion and residues from motifs I, II and VI (Fig. 4; (Xu et al., 2005)). The lack of specific amino acid interactions with the ATP nucleobase or ribose ring makes it challenging to design inhibitors to interact with this pocket. The crystal structure of DENV helicase–RNA–AMPPNP ternary complex (Luo et al., 2008a,b) showed that the RNA ligand binds to a shallow pocket on the helicase (Fig. 4), making it challenging to design compounds to inhibit RNA–protein interaction.

Despite the availability of a high-resolution structure of the DENV helicase, limited progress has been made towards the rational design of helicase inhibitors. Recently, an *in silico* docking of compounds into the single-strand RNA access site of WNV helicase identified ivermectin (Table 3) which inhibited WNV, YFV, and DENV helicase dsRNA unwinding activity at submicromolar levels. However, ivermectin did not inhibit the NS3 ATPase activity (Mastrangelo et al., 2012). When tested in cell culture, ivermectin inhibited YFV with $EC_{50}$ of 0.5 nM, but was less potent against DENV ($EC_{50}$ 0.7 μM), JEV ($EC_{50}$ 0.3 μM), WNV ($EC_{50}$ 4 μM), and TBEV ($EC_{50}$ 0.2 μM) (Mastrangelo et al., 2012). As mentioned above, ivermectin also inhibits DENV protease activity (Tomlinson and Watowich, 2012); therefore, the observed anti-DENV activity in cell culture could be the inhibition of both protease and helicase activities. Ivermectin is a broadly used anti-helminthic drug. It remains to be determined whether *in vivo* efficacy could be achieved in DENV.

NITD performed one HTS using an *in vitro* unwinding assay. We failed to identify specific helicase inhibitors. After counter-screens, the HTS hits were mostly found to be assay artifacts or nonspecific binding to the RNA substrate. These challenges are shared by other researchers working on other viral or host helicases. Readers are encouraged to refer to an excellent review on targeting helicases for drug development (Shadrick et al., 2013). For the DENV helicase, the need to design inhibitors that do not cross-react with host helicases is another hurdle, given the large number of host helicases in the SF2 superfamily (Lescar et al., 2008).

Byrd et al. (2013b) recently reported a novel small molecule inhibitor (ST-610; Table 3) of the DENV helicase. The compound was identified from an HTS using a DENV infection assay. ST610 potently and selectively inhibited all four serotypes of DENV in cell culture. Sequence analysis of drug-resistant virus found a single amino acid change (A263T) in the helicase domain that confers resistance to the compound. ST-610 inhibits DENV helicase RNA unwinding activity, but does not inhibit its ATPase activity. When dosed intraperitoneally (up to 100 mg/kg BID), ST-610 showed marginal efficacy in the DENV AG-129 mouse model, with <10-fold viremia reduction. For further development, the *in vivo* pharmacokinetic properties of this compound need to be improved.

## 11. Methyltransferase inhibitors

The N-terminal domain of the flavivirus NS5 protein (amino acids 1–272) functions as a dual methyltransferase (MTase), which can methylate the 5′-end of the viral genomic RNA at the N-7 position of the guanine cap (N-7 MTase) as well as the 2′-OH position of

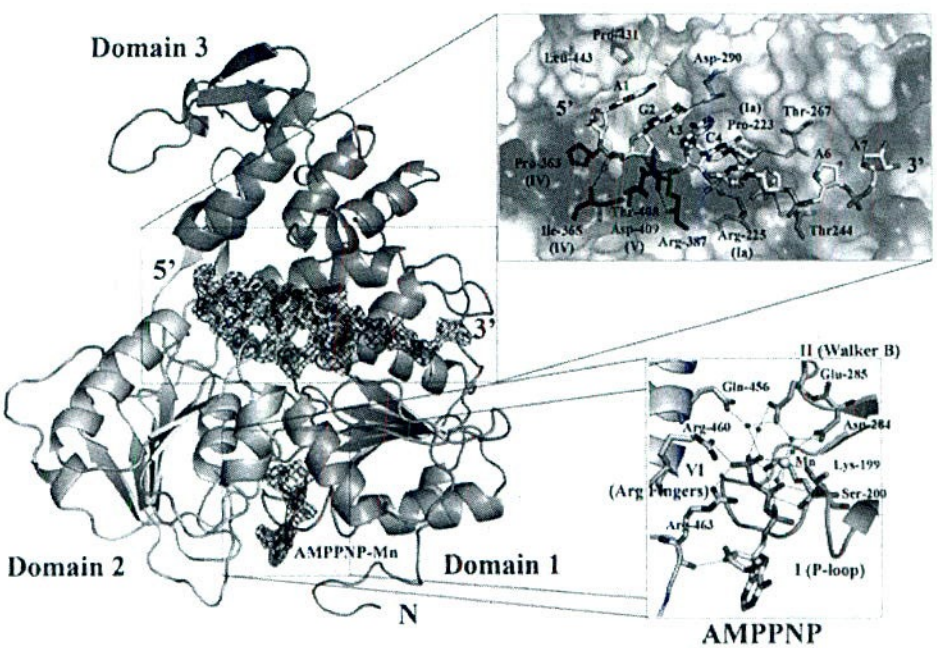



**Fig. 4.** The NENV helicase–RNA–AMPPNP ternary complex. Cartoon representation of NS3 helicase bound to ssRNA and AMPPNP, a non-hydrolysable ATP analog (AMPPNP) shown as sticks (Luo et al., 2008a,b). Domain 3 of the helicase is colored in orange. The Rec-A-like subdomains 1 and 2 are colored in green and blue, respectively. The manganese ion is represented as a yellow sphere. Top inset: magnified view of interactions between the protein and its RNA ligand (5′-AGACUAA-3′), using a color-coded representation of the helicase electrostatic surface potential. The color scale is 10 kT (red) to þ10 kT (blue). Residues that contact the RNA are displayed as sticks and labeled. Bottom insert: magnified view of the AMPPNP-$Mn^{2+}$ ternary complexes (with bound RNA). Water molecules are shown as red spheres. Hydrogen bonds are indicated by dashed lines.

the first nucleotide ribose (2′-O MTase) (Egloff et al., 2002; Ray et al., 2006). It can further methylate internal adenosines of the viral RNA genome and potentially host RNA at the ribose 2′-OH position (internal methylation) (Dong et al., 2012). Both the cap and internal methylations use *S*-adenosine-L-methionine (SAM) as the methyl donor and generate *S*-adenosine-L-homocysteine (SAH) as a by-product. Despite exhibiting two distinct methylation activities, flavivirus MTases show a single binding site for SAM in the apo-crystal structure and display high homology to host MTase (Noble and Shi, 2012).

Functional studies showed that N-7 methylation of RNA cap is critical for efficient translation (Ray et al., 2006). Mutations of MTase that abolish N-7 methylation are lethal for flavivirus replication (Zhou et al., 2007), suggesting that the N-7 MTase is a potential antiviral target. 2′-O cap methylation functions to subvert innate host antiviral response through escape of IFIT-mediated suppression (Daffis et al., 2010).

The wealth of structural information has provided a solid foundation for structure-guided antiviral approaches. However, since the core domains of various MTases are conserved, designing flavivirus-selective inhibitors which do not inhibit host MTases, e.g., RNA and DNA MTase or protein MTase, or SAM-binding proteins, will be challenging. Using a structure-guided approach, we recently designed DENV-selective inhibitors by taking advantage of a flavivirus MTase-unique pocket located next to the SAM binding site (Fig. 5; (Dong et al., 2010; Lim et al., 2011)). Specifically, SAM analogs (e.g., compound 10; Table 4) with an extra chemical group that interacts with the flavivirus-unique pocket selectively inhibited viral MTases. Although this provided proof of concept *in vitro*, the compound needs to be improved for cell permeability to achieve antiviral activity in cell culture.

Over the past three years, we have conducted two medium-throughput screens to identify inhibitors of the DENV MTase. Our first screen measured DENV N7 and 2′-O activities using the scintillation proximity assay (SPA) format as it is amenable to miniaturization (Chung et al., 2010; Lim et al., 2008). About 60,000 compounds were screened. After counter screens, we failed to identify any specific hits. It is possible that the relatively small pool of compounds tested did not contain any specific inhibitors and screening with a larger number of compounds would increase

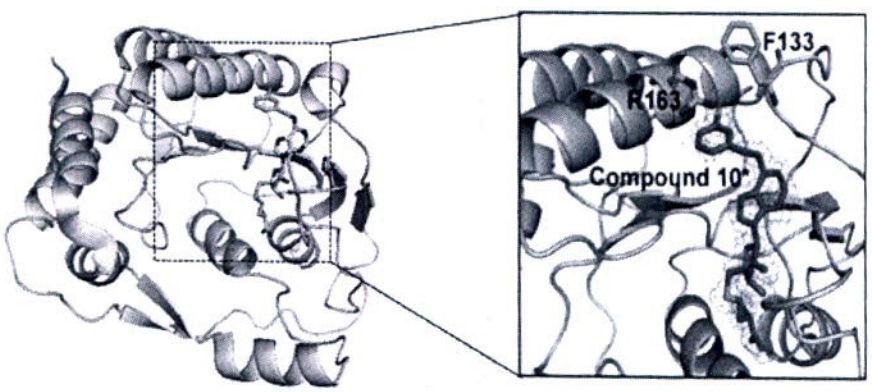
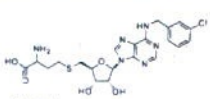
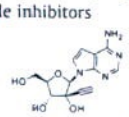
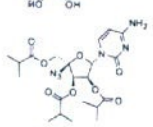
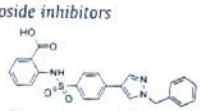
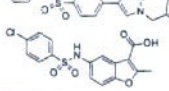



**Fig. 5.** Crystal structure of the DENV MTase in complex with compound 10. Surface representation of the DENV MTase shows the flavivirus-conserved hydrophobic cavity (yellow) and the bound compound 10 (in stick presentation). Inset magnifies compound 10 bound to the MTase gydrophobic pocket (in cartoon presentation); the confomation of amino acids Phe-133 and Arg-163 was changed upon binding to compound 10 (Lim et al., 2011).

**Table 4**
Inhibitors of DENV NS5 MTase and RdRp (see details in text).

| Compound | Structure | Mode of action | Cellular activity ($EC_{50}/CC_{50}$) μM | AG129 dengue mouse activity | References |
|---|---|---|---|---|---|
| *MTase inhibitor* | | | | | |
| Compound 10 |  | MTase | ND | ND | Lim et al. (2011) |
| RdRp nucleoside inhibitors | | | | | |
| NITD-008 |  | Adenosine nucleoside | 0.64/>100 | Efficacious | Yin et al. 2009a,b |
| Balapiravir |  | Cytidine nucleoside | 1.9/ND | ND | Nguyen et al. (2013) |
| *RdRp non-nucleoside inhibitors* | | | | | |
| NITD-2 |  | RdRp non-nucleoside | ND | ND | Niyomattanakit et al. 2010a,b |
| NITD-107 |  | RdRp non-nucleoside | ND | ND | Noble et al. (2013) |

the likelihood of success. However, extra screening was not carried out because the costs of the SPA beads, tritiated-labeled SAM, and the capped/biotinylated RNA substrates together were prohibitively high. Our second screen measured DENV 2′-*O* MTase activity using a fluorescent polarization assay (Lim, et al., 2013). We screened 250,000 compounds. After extensive follow-ups, no specific hits were identified. Considering the disappointing outcome of the enzyme activity-based screens, we believe that a structure-guided approach or perhaps a competitive binding assay (e.g. SPR or NMR) may be more suitable for hit identification for flavivirus MTase.

## 12. Polymerase nucleoside analog inhibitors

The NS5 polymerase is the most conserved flavivirus protein and is an attractive drug target, as it is essential for viral RNA synthesis. Readers are encouraged to refer to several excellent reviews on its structure and function (Bollati et al., 2010; Lescar et al., 2008; Malet et al., 2008; Noble and Shi, 2012).Viral polymerase inhibitors can be classified into two broad categories: nucleoside/nucleotide analog inhibitors (NIs) and non-nucleoside inhibitors (NNIs). NIs, when converted to the corresponding triphosphate, can compete with natural NTP substrates and be incorporated into the growing RNA chain to terminate elongation. Besides RNA chain termination as the main mode of action, some NIs, such as ribavirin, could be incorporated into an RNA chain without termination. When such an RNA chain serves as a template for RNA synthesis, the integrated NIs cause mutations in the daughter RNAs, leading to error catastrophe (De Clercq and Neyts, 2009). NNIs usually bind to allosteric sites in the polymerase; NNIs either "lock" the enzyme into an inactive form or prevent conformational changes required for initiation and/or elongation of RNA synthesis.

NIs represent the largest class of approved antiviral regimens in clinical use. There are clear therapeutic benefits with this class of inhibitors as seen in the treatment of viral diseases such as HIV-1 (Cihlar and Ray, 2010), and HBV infection (De Clercq, 2011). While conceptually easy, there are several key challenges in developing NIs for therapeutics. First, it is often impossible to predict the structure–activity relationship of the compounds, as several enzymatic steps are required to convert them to active triphosphates (Stein and Moore, 2001). This makes it challenging for classical medicinal chemistry approaches to improve potency. Therefore, potent NIs are often discovered serendipitously. Second, the toxicity of nucleoside analogs is unpredictable and is often missed *in vitro*. Although mitochondrial toxicity, such as inhibition of polymerase gamma, has been implicated in most antiviral NIs, especially for those for the treatment of HIV-1 infection, there are other toxicities associated with NIs (Dagan et al., 2002). Thus, animal toxicological studies are often needed to assess the potential side effect of this class of compound, which is often costly in terms of compound and animal requirement. Our experience with NITD-008 (Table 4), an adenosine analog which potently inhibited DENV replication, is a good example (Chen et al., 2010a; Yin et al., 2009b). Treatment of DENV-infected mice with NITD-008 suppressed peak viremia and completely prevented death in a lethal mouse model. No adverse effect was observed when rats were orally dosed with NITD-008 at 50 mg/kg/day for 1 week. However, after 2 weeks of oral dosing, severe side-effects were observed in both rats and dogs. These results led to the termination of NITD-008 for further development for DENV treatment.

There are several advantages which make NIs attractive for drug development. For instance, since NIs target polymerase active sites which are often conserved, it has a high barrier of resistance emergence compared to other classes of inhibitors (Delang et al., 2011). Moreover, NIs usually display equivalent potency against different serotypes/genotypes of a particular virus and even related viruses (Kuntzen et al., 2008). For example, several NIs synthesized by NITD have shown equipotency against all four serotypes of DENV as well as other closely related flaviviruses (Chen et al., 2010a,b; Yin et al., 2009b). Another example is tenofovir, which is effective in both HIV and HBV (Woo et al., 2010). Similarly, we (Yin et al., 2009b) and others (Migliaccio et al., 2003; Nguyen et al., 2013) have observed cross-reactivity of NIs against DENV and HCV.

Balapiravir (Table 4) is the tri-isobutyrate ester prodrug of 4′-azidocytidine (R1479). It was originally developed for HCV treatment by Hoffmann-La Roche. Unfortunately, its clinical development for HCV was terminated due to hematological adverse effects in patients after extended treatment (Nelson et al., 2012). Since R1479 has anti-DENV activity, it was repurposed for a phase II trial for DENV (Nguyen et al., 2013). Dengue patients received balapiravir at doses of 1500 mg ($n = 10$) or 3000 mg ($n = 22$) orally for 5 days. The patients were recruited based on the following criteria: (i) fever with temperature $\geqslant 38$ °C; (ii) a positive NS1 rapid test; (iii) onset of symptoms <48 h prior to initial dosing. Surprisingly, balapiravir did not improve the clinical and virological parameters in patients, including viral levels in the blood, NS1 production, as well as fever duration. The reason for the lack of efficacy remains to be determined. *In vitro* activity of balapiravir against DENV ranged from $EC_{50}$ values of 1.9–11 μM and 1.3–3.2 μM in DENV HuH7 replicon and human PBMC DENV infection assays, respectively. Both doses administered to DENV patients attained plasma drug levels comparable to the $EC_{50}$ values ($C_{max}$ >5.5 and 8.8 μM with 1500 mg and 3000 mg, respectively). It was speculated that perhaps higher plasma levels of the drug are needed to achieve antiviral effects.

Besides the challenges and opportunities described above, the following three categories of parameters make NIs uniquely different from other classes of viral inhibitors.

### 12.1. Dissolution, absorption, and cellular uptake

NIs are usually quite polar, and thus solubility is usually not an issue for this class of inhibitor, unless large amounts of drug are needed for efficacy. However, a highly polar surface area may not be ideal for cellular uptake; the addition of simple ester (prodrugs) usually improves absorption and exposure (Brandl et al., 2008). Cellular uptake is usually via passive diffusion, which may not be optimal for this class of compounds. In some cases, they pass through the plasma membrane via nucleoside transporters (Cass et al., 1999). The highly polar surface area also makes them unlikely to penetrate the blood–brain barrier. Thus, the use of this class of drugs in viral encephalitis could be limited.

### 12.2. Metabolism

When dosed as nucleoside analogs, NIs are inactive without transformation into the corresponding triphosphates intracellularly. As a result, their activity can only be evaluated using cellular assays. In addition, due to differences in the expression of enzymes responsible for converting the nucleoside into its active nucleoside triphosphates, they are prone to cell-type variations. It is therefore essential to evaluate these drugs using relevant target cells. For developing DENV NIs, it is also advisable to profile them in physiologically relevant cells, such as hematopoietic cells, where DENV replicates in patients (Noisakran et al., 2010). However, the dependency on cellular activation could be advantageous if the target organ is rich in enzymes which convert the drug into its triphosphate active form. This avenue is indeed employed to target HCV NIs to the liver (McGuigan et al., 2010), or alternatively to use CYP enzymes as the first activation step of a prodrug (Niu et al., 2012).

The conversion of a nucleoside analog into corresponding monophosphate is often the rate-limiting step in nucleoside activation (Stein and Moore, 2001). However, in a few cases, such as zidovudine, the conversion of zidovudine monophosphate into the diphosphate by thymidylate kinase is the rate-limiting step (Lavie et al., 1997). To circumvent the first phosphorylation dependency, newer prodrugs often have pre-attached monophosphates or phosphoramidate/ester which could greatly improve potency (Zhou et al., 2011). The pre-attached monophosphate brings in extra complications, because the monophosphate moiety is very polar, making the final molecule more inefficient to cross plasma membrane. Additional masking groups are needed to protect the monophosphate moiety and to improve plasma membrane penetration; these masking groups have to stay intact throughout the absorption and distribution phase, but should be broken down quickly once the drug reaches the target organ. Since intracellular concentration of modified nucleoside triphosphate is the active inhibitor for viral polymerase, the intracellular level of the triphosphate nucleoside is expected to correlate with the level of viremia reduction. This concept was proved in AZT-treated HIV patients (Fletcher et al., 2000). Along the same line, the pharmacokinetics of the triphosphate nucleoside, rather than plasma exposure of the nucleoside, is a better guide for the dosing regimen (Wang et al., 2004).

### 12.3. Factors affecting efficacy of the drug

Different cells express a diverse range of enzymes responsible for nucleoside triphosphate conversion; thus, the potency of the nucleoside will not be the same. In addition, factors such as concentration of natural nucleoside triphosphate (Garcia-Lerma et al., 2011) and activation cell state (Gao et al., 1993) can influence the efficacy of NIs as well. A special type of drug-drug interaction occurs when two nucleosides share a common activation pathway (Veal et al., 1997). These factors should be considered in combination when evaluating nucleoside inhibitors.

## 13. Polymerase non-nucleoside inhibitors

NNIs are typically non-competitive inhibitors. They act by occupying specific pockets in the polymerase, and block conformational changes which are needed for its initiation and/or elongation activities. While there are many good examples of NNIs targeting HIV-1 reverse transcriptase in clinical use (Blas-Garcia et al., 2011), there are as yet no FDA-approved NNIs available for other viral polymerases. Numerous HCV RdRp NNI drug candidates (Sofia et al., 2012) have been tested in clinical settings, but the results, such as for tegobuvir (Gilead; indirect polymerase inhibitor) or filibuvir (Pfizer; thumb II site inhibitor), have been disappointing, as no efficacy was observed as monotherapy (Pockros, 2013). Other compounds, such as setrobuvir (ANA598; palm I inhibitor), BI207127 (thumb I site inhibitor), or VX-222 (thumb II site inhibitor) are currently in phase II trials.

There are several potential drawbacks to the use of NNIs in antiviral therapy. First, the pocket must be well conserved for the compound to be broadly acting across different serotypes or genotypes. Second, the virus may generate amino acid mutations in or near the NNI binding pocket to overcome its inhibitory action. Alternatively, naturally occurring resistant viruses may exist in circulation which are insensitive to a particular NNI, resulting in the expansion of "escape mutants." For these reasons, DENV RdRp NNIs are envisaged to be administered in combination with another drug with a high barrier to resistance (either an NI or another DAA) to prevent the emergence of resistant viruses. On the positive side, highly specific NNIs can translate to lower off-target effects and potentially lower cytotoxicity. This is boosted by the fact that the host has no RdRp protein.

Most first-generation NNIs are often identified through high-throughput screening activities using RdRp biochemical assays. However, during hit-to-lead optimization, obtaining RdRp co-crystal structures with bound inhibitors opens up opportunities to further develop analogs via a structure-guided approach (Melagraki and Afantitis, 2011). Alternatively, strategies such as fragment-based screening may also be considered (Scott et al., 2012). On this note, few allosteric binding pockets are present in the "closed" conformation of the DENV RdRp (Malet et al., 2008; Zou et al., 2011). It remains to be seen if additional pockets would be formed in the "open" conformation upon RNA binding. This information awaits the co-crystal structure of the DENV RdRp–RNA complex.

Since one of the major issues related to NNIs is the likelihood of emergence of resistant viruses, understanding the mode of action and improving the binding mechanism would help to reduce this problem. NNIs that have strong inhibitory effects on enzyme catalysis, as well as high binding affinity with good kinetic profiles (e.g., fast $K_{on}$, slow $K_{off}$, and a long residence time) will make better candidates (Winquist et al., 2013).

NITD conducted two HTS campaigns, both screening about 1.8 million compounds, using biochemical assays that measured DENV polymerase elongation activities. The first HTS used an SPR RdRp elongation assay. We identified one compound class (e.g., NITD-2; Table 4) that bound in the RNA tunnel of the RdRp, and likely functions by blocking RNA synthesis (Niyomrattanakit et al., 2010b). Although good SAR was observed from analogs tested in the elongation SPA assay (Yin et al., 2009a), the series could not be advanced due to poor cellular permeability. The second HTS utilized a fluorescent-coupled elongation assay (Niyomrattanakit et al., 2010a). Unfortunately, many of the hits identified from this HTS failed to exhibit binding specificity to DENV RdRp. A few hits that inhibited the enzyme were exemplified by NITD-107 (Table 4; (Noble et al., 2013)). Although NITD-107 binds in the DENV RdRp active site (Fig. 6), it showed only weak inhibitory activity and a flat SAR during chemistry campaign (unpublished). In both HTS, there was a substantial number of false positives which acted by aggregating the protein or interfering with the assay readout. One possible reason for this finding was the poor stability of the recombinant DENV polymerase, making the enzyme sensitive to nonspecific inhibition. Alternatively, the fraction of catalytically active DENV RdRp in the total protein population may be very low, making it susceptible to this problem. Indeed, for HCV, the proportion of active form in the recombinant protein has been reported to be <1% (Carroll et al., 2000). In addition, since the polymerase requires divalent ions to function, the assay is sensitive to inhibition by metal chelators. Improving the stability or specific activity of recombinant DENV RdRp would certainly help to overcome these challenges.

## 14. Host target inhibitors

Targeting host factors required for the virus life cycle, instead of virus-encoded proteins, opens new perspectives to counteract viral infections and is being applied to different families of viruses (Schwegmann and Brombacher, 2008; Tan et al., 2007). For example, there are currently a few phase I and II HCV clinical trials in which the compounds target host proteins, such as cyclophilin, TLR (agonist), histone deactylase and caspases. This approach offers the advantage of a significantly higher barrier to the emergence of resistance, compared with DAA, since viral mutations are less able to compensate for the loss of an essential host cofactor. Furthermore, different viruses may share a host

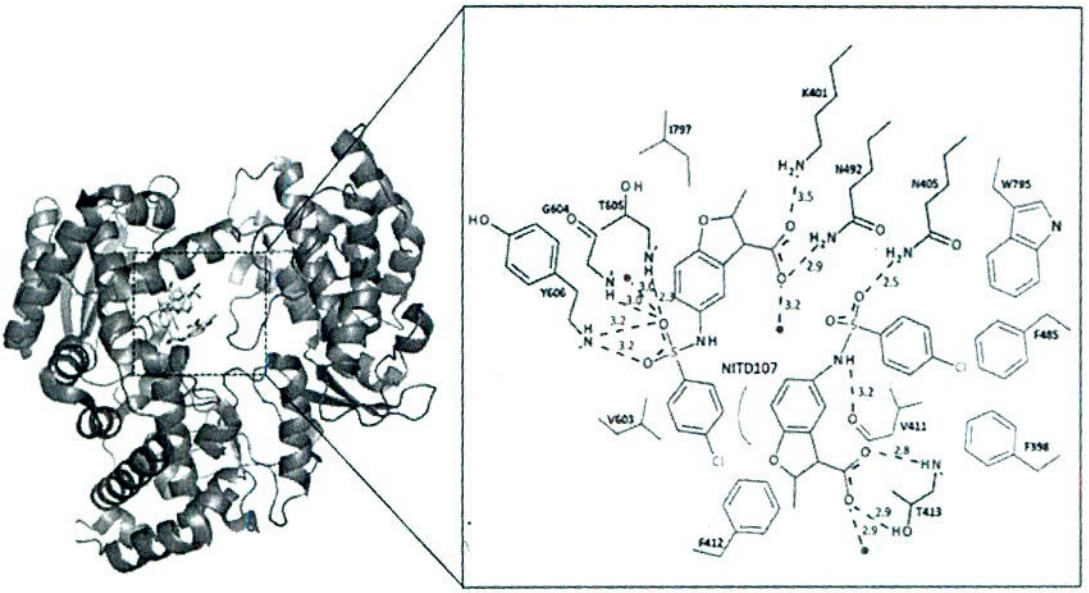
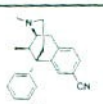
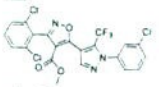
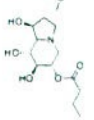



**Fig. 6.** Crystal structure of DENV RdRp bound to two molecules of NITD-107 (Noble et al., 2013). The protein is shown as a cartoon and the compound molecules as sticks. Boxed section shows the map of amino acid interaction between RdRp and NITD107 dimer. Interactions between RdRp and NITD107 dimer. Interatomic distances between 2.5 to 3.2 Å are depicted.

**Table 5**
Inhibitors of host targets (see details in text).

| Compound | Structure | Mode of action | Cellular activity ($EC_{50}/CC_{50}$) μM | AG129 dengue mouse activity | References |
|---|---|---|---|---|---|
| NITD-451 |  | Translation inhibitor | 0.16/57 | Efficacious | Wang et al. (2011b) |
| NITD-982 |  | Inhibit pyrimidine biosynthesis through DHODH | 0.0024/>5 | Not efficacious | Wang et al. (2011a) |
| Celgosivir |  | Alpha-glucosidase | 0.22/>100 | Efficacious | Rathore et al. (2011), Schul et al. (2007), Whitby et al. (2005) |

factor/pathway for replication, so that compounds targeting the shared host factor/pathway could have pan-antiviral activities.

The drawback of targeting host factors is the higher potential for undesirable drug-induced side effects, because these factors are often essential for cell survival or metabolism. However, this approach would encounter fewer hurdles in the treatment of acute diseases, such as dengue, compared with the treatment of chronic diseases like HIV-1 or HCV infection (Schang, 2006). Several host pathways have been shown to be important for DENV replication, including the ubiquitin–proteasome system (Fink et al., 2007; Kanlaya et al., 2010), the unfolded protein response (Umareddy et al., 2007; Sessions et al., 2009) and lipid metabolism (reviewed in Fischl and Bartenschlager, 2011; see also below).

### 14.1. Viral translation inhibtors

Screening of the Novartis compound library using a DENV-induced cytopathic (CPE) assay led to the identification of a class of compounds with a benzomorphane core as inhibitors of DENV (Wang et al., 2011b). The initial "hit" NITD-2636 exhibited 76% cell protection at 5 μM. NITD-2636 displayed a broad spectrum of antiviral activity. It not only reduced the titer of DENV, but also the titers of other flaviviruses, including YFV and WNV. However, the compound did not efficiently suppress non-flaviviruses such as the alphavirus WEEV or VSV. Synthetic chemistry efforts were carried out using NITD-2636 as a starting point to explore the structure–activity-relationship (SAR), with the aim of improving metabolic stability. Substitution of the metabolic-labile phenolic-OH group with an electron-withdrawing cyano group (NITD-451; Table 5) retained antiviral activity while improving stability.

The mechanism of action of NITD-451 (Table 5), was elucidated using a DENV luciferase-reporting replicon system. NITD-451 suppressed DENV driven luciferase activity as early as 2 h post-transfection, indicating its inhibitory role in RNA translation. Its mode of action was further validated with a cell-free translation system, in which *in vitro* translation of a reporter RNA was directly suppressed by the compound. As DENV uses host machinery for translating its genome, NITD-451 targets a host factor involved in translation. The identity of this factor remains to be determined. The interaction between the compound and its target is specific, as only the S,R,S enantiomer NITD-451 was active in inhibiting DENV replication and RNA translation, whereas the R,S,R

enantiomer NITD-452 was inactive in both assays. *In vivo* efficacy experiments showed that treatment of DENV-infected mice with 25 mg/kg of NITD-451 reduced peak viremia by about 40-fold, without any obvious adverse effects. However, mice treated with 75 mg/kg of compound per day exhibited adverse effects, thus terminating the progression of this class of compound.

Similar to the DENV translation inhibitor, Noueiry et al. (2007) identified compound AP30451that selectively inhibits flavivirus RNA translation. The compound was identified through library screening, using a WNV replicon assay (Noueiry et al., 2007).

### 14.2. Host pyrimidine biosynthesis inhibitors

In the CPE-based HTS campaign mentioned above, another interesting class of compounds with an isoxazole-pyrazole core was identified (Wang et al., 2011a). One such compound, NITD-982 (Table 5), displayed nanomolar potency against four different RNA virus families, including the *Flaviviridae*, *Paramyxoviridae*, *Orthomyxoviridae*, and *Retroviridae*. The broad-spectrum activity of this compound strongly indicated that a cellular target was involved in its mechanism of action.

To identify the cellular target(s) of NITD-982 (Table 5), a three-channel iTRAQ quantitative chemical proteomics technology was implemented (Bonavia et al., 2011). NITD-102, a bioactive analog of NITD-982, was synthesized and immobilized to a column to capture target protein from virus-infected cell lysates. The protein identified was dihydroorotate dehydrogenase (DHODH), which is a mitochondrial protein that catalyzes the oxidation of dihydroorotate to orotate, the fourth enzymatic step of the *de novo* pyrimidine biosynthesis. An integral approach of biochemical, biophysical, and pharmacological techniques was used for target validation. The results showed that

(i) The compound inhibits the enzymatic activity of the recombinant DHODH;
(ii) an analog of NITD-982 directly binds to the recombinant DHODH;
(iii) the compound-mediated inhibition of viral replication could be reversed by supplementing culture medium with uridine; and
(iv) DENV-2 variants resistant to Brequinar (a known DHODH inhibitor) are cross-resistant to NITD-982.

Collectively, the results indicate that NITD-982 directly inhibits DHODH activity to deplete intracellular pyrimidine pools, leading to the suppression of viral RNA synthesis. However, its *in vitro* efficacy did not translate into *in vivo* efficacy. This lack of antiviral efficacy *in vivo* could be attributed to the uridine uptake from diets that replenish or maintain a high concentration of pyrimidine in the plasma, therefore counteracting compound-mediated inhibition of viral replication.

### 14.3. Host cholesterol synthesis pathway inhibitors

Cholesterol biosysnthesis and transport have been implicated for the replication of several flaviviruses, including DENV. Decreased levels of plasma cholesterol, lipoproteins, and triglycerides are associated with the severity of dengue disease (Villar-Centeno et al., 2008; van Gorp et al., 2002). Lee et al. (2008) found that disruption of lipid raft formation by cholesterol depletion with methyl-beta-cyclodextrin or cholesterol chelation with filipin III reduced JEV and DEN-2 infection, mainly at intracellular replication steps and, to a lesser extent, at viral entry. DENV infection was also impaired when cholesterol intake in infected cells was disrupted using a cholesterol transport inhibitor, U18666A (Poh et al., 2009). The compound negatively affected viral trafficking in the cholesterol-loaded late endosomes/lysosomes, as well as *de novo* sterol biosynthesis. An additive antiviral effect of U18666A with C75, a fatty acid synthase inhibitor, was observed, suggesting that DENV relies on both cholesterol and fatty acid biosynthesis for its successful replication. In addition, hymeglusin and zaragozic acid A (inhibitors of HMG-CoA synthase and squalene synthase, respectively) inhibited DENV2 NGC replication in K562 cells, while lovastatin (HMG-CoA reductase inhibitor) reduced its replication in human peripheral blood mononuclear cells (Rothwell et al., 2009).

### 14.4. Cellular glucosidase inhibitor: Celgosivir

Celgosivir (butyl-castanospermine; Table 5) is an oral prodrug of the natural product castanospermine that inhibits alpha-glucosidase I, an enzyme that plays a critical role in viral maturation by initiating the processing of N-linked oligosaccharides of viral envelope glycoproteins. It was tested in a phase II clinical trials in combination with peglated IFN and ribavirin for treatment of HCV infection. It was not further pursued, as it was not better than the standard drug regimen (Durantel, 2009).

Several studies have indicated that castanospermine and Celgosivir also inhibit DENV replication, disrupting the folding of the DENV structural proteins prM and E (Whitby et al., 2005) as well as NS1 (Rathore et al., 2011). Intraperitoneal injection of castanospermine prevented mortality in an intracranial lethal challenge mouse model (Whitby et al., 2005) and significantly reduced plasma virus levels in a viremia mouse model, when dosed orally at 75 mg/kg twice a day (Schul et al., 2007). Subsequent testing of Celgosivir also indicated good efficacy in a lethal ADE dengue infection mouse model. Twice-a-day dosing at 50 mg/kg via intraperitoneal injection for 5 days completely protected all mice from death (Rathore et al., 2011). Treatment with 25 mg/kg BID of Celgosivir was comparable to 50 mg/kg BID of castanospermine, suggesting that the prodrug was twice as efficacious, very likely due to its better cellular permeability (Watanabe et al., 2012).

Based on these encouraging findings, a phase Ib clinical trial is currently ongoing to evaluate the activity, pharmacokinetics, safety, and tolerability of Celgosivir in dengue patients. Using PK simulations, it was anticipated that 200 mg BID in humans would give rise to a similar steady-state $C_{min}$ as 50 mg/kg BID in mice (Watanabe et al., 2012). The efficacy results of Celgosivir in dengue patients will impact on the development of other alpha-glucosidase inhibitors. Besides Celgosivir, other iminosugar analogs targeting the host alpha-glucosidase have been shown to be efficacious against DENV, both in cell culture and the AG129 mouse model (Miller et al., 2012; Yu et al., 2012).

A previous trial in HIV-1 patients identified grade III toxicity in transaminase, creatine kinase (with normal CkMB isoenzyme) and LDH after 14 days of oral dosing at 300 mg BID; similar toxicity was observed after 14 days of oral dosing at 450 mg QD (Roth et al., 1996). Consequently, the dosing regime for dengue patients comprised a loading dose of 400 mg, followed by 200 mg BID for a total of 5 days. The inclusion criteria are similar to the balapriravir trial conducted on DENV patients (Nguyen et al., 2013).

### 14.5. Severe dengue disease inhibitors

Patients with dengue exhibit a variety of serious complications, including plasma leakage, hypovolemic shock, thrombocytopenia and bleeding. These occur as plasma viremia is resolving, and are consequently thought to be virus-driven immunological responses (Libraty et al., 2002a,b). Inhibitors of immunopathological pathways could broaden the window of treatment time, especially for treatment of patients in whom the viremia duration is short. Unfortunately, the molecular details of the host pathways that lead

to severe dengue (DHF/DSS) are not well defined; the feasibility of such an approach therefore remains low at this time. However, this approach was recently explored by two studies.

Tam and co-workers (2012) conducted corticosteroid therapy in early infected dengue patients. Corticosteriods are potent inhibitors of inflammatory processes and suppress the production of cytokines, chemokines, arachidonic acid metabolites and adhesion molecules. No improvement of clinical and virological parameters was observed in the corticosteroid-treated patients, compared to the placebo control group. St John et al. (2013) reported that treatment of DENV-infected mice with mast cell-stabilizing drugs or a leukotriene receptor antagonist restored vascular integrity, suggesting that these compounds could be evaluated for their effectiveness in improving disease outcomes in viral hemorrhagic fevers. However, the drug-treated animals exhibited a slight increase of viremia; this phenomenon should be carefully monitored during further development.

## 15. Conclusions and perspectives

Public–private partnership is an attractive model to raise resources for combating neglected diseases. Building on this model, NITD has made significant contributions to dengue biology and drug discovery, through fruitful collaborations with research institutes in Singapore and leveraging expertise available in Novartis research entities. Studies in the collaborative EDEN program generated insights into dengue diagnosis, epidemiology and pathology, through detailed clinical analyses of dengue patients (Christenbury et al., 2010; Schreiber et al., 2009; Tanner et al., 2008; Fink et al., 2007). In collaboration Julien Lescar's group, the NITD contributed to the first crystal structures of the DENV helicase (Xu et al., 2005), full-length NS3 (Luo et al., 2008a,b), and RdRp (Yap et al., 2007). The first catalytically active flavivirus protease structure (represented by WNV protease) was solved together with Novartis colleagues in Basel (Erbel et al., 2006). These structures, together with the structures of other DENV proteins or the same proteins of other flaviviruses solved by various academic groups (Egloff et al., 2002; Li et al., 2008; Ma et al., 2004b; Malet et al., 2007; Modis et al., 2003, 2004; Rey et al., 1995; Wu et al., 2005) have tremendously enabled DENV drug discovery.

As the only institute with a dedicated mission to develop dengue antivirals, the NITD has taken a concerted approach for dengue drug discovery. Although the effort has not yet generated a clinical candidate, the experience accumulated during the past decade has provided a better rationale for the on-going anti-DENV effort. Compared with the resources that have been invested in HCV antiviral development during the past 25 years, those spent on dengue have been much smaller.

Two general strategies can be pursued for any antiviral therapy. The first is to inhibit viral targets; such an approach has generated most of the DAAs currently in clinical use. The second is to inhibit host targets. Two types of host targets could be pursued: (i) host factors that are essential for viral replication. As exemplified by the pyrimidine biosynthesis inhibitor, targeting host factors has extra unknown hurdles, compared with targeting viral factors. (ii) Host targets that participate in the development of disease symptoms. As mentioned before, inhibitors of pathological pathways could broaden the window of treatment time; this is especially critical for the successful treatment of dengue patients, in whom the duration of viremia is short. Unfortunately, the molecular details of host pathways that lead to DHF/DSS are not well defined; therefore, the feasibility of such an approach remains low at this time. However, targeting host factors has the advantage of a high resistance barrier, as exemplified by HCV compounds that inhibit cellular cyclophilin (Puyang et al., 2010).

One bottleneck for dengue drug discovery is knowledge about sites of viral replication in patients, which is critical for designing compounds that can selectively deposit to replication sites. It is generally accepted that the major replication sites during DENV viremia are monocytes, macrophages, and dendritic cells (Durbin et al., 2008; Rothman, 2011). However, autopsies of a few dengue patients detected viral antigen in hepatocytes (Rosen et al., 1999) and Kupffer cells (Jessie et al., 2004). It remains to be determined whether the postmortem autopsy results are relevant to the current antiviral rationale of suppressing viremia during early infection to achieve clinical benefits. Therefore, for DAA to be effective, they should be able to target sites where early virus replication occurs.

Since the amino acids homology among the four serotypes of DENV is about 70% (Lindenbach et al., 2007), it could be challenging to design a compound that potently inhibits all of them. This goal can be achieved by nucleoside inhibitors, because they interact with the flavivirus-conserved active site of the RdRp during RNA synthesis. For the same reason, a nucleoside analog that is active against DENV is likely to be active against other flaviviruses. For other types of DAA, the antiviral spectrum against all DENV serotypes should be closely monitored during hit characterization. In analogy to HCV antiviral therapy, developing a compound efficacious against all four serotypes of DENV is as challenging as the development of a compound that is active against all six genotypes of HCV (with amino acid variation of about 30%).

In summary, great progress has been made in DENV biology and antiviral drug discovery in the past decade. Many lessons have been learned from various approaches. Though challenging, we are optimistic that the continuing effort will lead to an effective anti-DENV therapy in the near future.

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# Chutes and Ladders in Hepatitis C Nucleoside Drug Development§

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## Abstract

Chutes and Ladders is an exciting up-and-down-again game in which players race to be the first to the top of the board. Along the way, they will find ladders to help them advance, and chutes that will cause them to move backwards. The development of nucleoside analogs for clinical treatment of hepatitis C presents a similar scenario in which taking shortcuts may help quickly advance a program, but there is always a tremendous risk of being sent backwards as one competes for the finish line. In recent years the treatment options for chronic hepatitis C virus (HCV) infection have expand due to the development of a replicon based *in vitro* evaluation system, allowing for the identification of multiple drugable viral targets along with a concerted and substantial drug discovery effort. Three major drug targets have reached clinical study for chronic HCV infection: the NS3/4A serine protease, the large phosphoprotein NS5A, and the NS5B RNA-dependent RNA polymerase. Recently, two oral HCV protease inhibitors were approved by the FDA and were the first direct acting anti-HCV agents to result from the substantial research in this area. There are currently many new chemical entities from several different target classes that are being evaluated worldwide in clinical trials for their effectiveness at achieving a sustained virologic response (SVR) (Pham et al., 2004; Radkowski et al., 2005). Clearly the goal is to develop therapies leading to a cure that are safe, widely accessible and available, and effective against all HCV genotypes (GT), and all stages of the disease. Nucleoside analogs that target the HCV NS5B polymerase that have reached human clinical trials is the focus of this review as they have demonstrated significant advantages in the clinic with broader activity against the various HCV GT and a higher barrier to the development of resistant viruses when compared to all other classes of HCV inhibitors.

### Keywords

Hepatitis C virus; HCV; nucleoside analog; nucleotide analog; antiviral; clinical evaluation; prodrug; clinical trials; nucleosides

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## 1. Introduction

The hepatitis C virus (HCV) was identified in 1989 as a member of the *Flaviviridae* family of positive-stranded RNA virus (Choo et al., 1989). Infection of a human host by HCV results in a serious infection affecting about 3% of the worldwide population, according to the World Health Organization (WHO). The WHO also estimates that 4 million people contract HCV each year (WHO, 2012). Although the early stages of HCV infection are usually asymptomatic, and about 20% of infected individuals will naturally clear the virus, a majority of infections progress to chronic infection. A significant number of chronically infected individuals will eventually develop more serious liver problems such as cirrhosis, hepatocellular carcinoma (HCC), or liver failure requiring liver transplantation (Darby et al., 1997; Poynard et al., 2000; Tong et al., 1995). The global health burden of HCV-associated morbidity and mortality is expected to increase substantially during the next decades, as many chronically infected individuals progress to end stage disease with associated complications (Davis et al., 2003; Manns et al., 2007). In fact, in industrialized nations, HCV infection is already the leading cause for liver transplantations. Unfortunately, re-infection of the transplanted liver from an unknown reservoir often occurs post-transplantation (Hoofnagle, 1997). The virus is transmitted parenterally by contaminated blood most often from sharing contaminated needles, but also from improper sterilization of medical, dental, body piercing, or tattoo equipment. Heterosexual transmission and vertical transmission (infected mother to her child during the birthing process) of HCV can also occur, but are rare (Fishman et al., 2009; Ghosn et al., 2009). Sexual practices that involve higher levels of trauma to the anogenital mucosa or that occur when there is a concurrent sexually transmitted infection, such as HIV or genital ulceration, do present a higher risk of HCV transmission (Tohme and Holmberg, 2010).

Three major drug targets have reached evaluation in humans for chronic HCV infection (Farnik and Zeuzem, 2012; Schaefer and Chung, 2012): the NS3/4A serine protease, the large phosphoprotein NS5A and NS5B RNA-dependent RNA polymerase (RdRp) (Bobeck et al., 2010; De Francesco and Migliaccia, 2005; Huang et al., 2006). The majority of drugs that target the HCV NS3/4A serine protease are peptidomimetics and represent the only two FDA approved direct acting antiviral agents for treatment of HCV infection. Although the ultimate role of NS5A in the HCV replication cycle is not fully understood, the 49 kDa NS5A protein is required for HCV replication as it is part of the membrane bound replication complex (Lemon et al., 2010). The two main categories of HCV NS5B RdRp inhibitors are non-nucleoside and nucleoside analog inhibitors (Brown, 2009; Burton and Everson, 2009; Legrand-Abravanel et al., 2010), which differ in their chemical structure, barrier to resistance, pan-genotypic activity and mode of action.

The current FDA-approved treatments for chronic HCV are limited to pegylated interferon-α (**IFN**) alone or in combination with ribavirin (**RBV**) with or without protease inhibitors (PI) boceprevir (Victrelis®) and telaprevir (Incivek®) (Fig. 1) (Hughes and Shafran, 2006; Pan et al., 2012; Sheridan, 2011). Non-pegylated **IFN**s have also been approved including the recombinant **IFN** alfacon1 and natural **IFN**, purified from the leukocyte fraction of human blood, multiferon. Unfortunately, these treatments have limited efficacy for the GT1 population, which is the most prevalent GT in the US and China and emphasize the urgent unmet medical need for new improved therapeutic agents that are pangenotypic (Manns et al., 2001; McCown et al., 2008). These treatments also have major side effects including depression, low red and/or white blood cell counts, central nervous system toxicity, fatigue, headache, flu-like symptoms, mild alopecia, thyroid dysfunction, itching, swelling of the face, eyes, lips, tongue, or throat, skin rashes, hemorrhoids, diarrhea and teratogenic effects (Fried, 2002; Gaetano and Reau, 2013; Gordon and Keller, 2005; Hezode et al., 2012;

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Keicher et al., 2005; Koch and Narjes, 2007; McHutchison et al., 2002; Pawlotsky, 2003; Wong and Lee, 2006).

In the clinic, the primary treatment goal for chronically infected HCV (CHC) persons is lifelong clearance of detectable HCV infection. When new anti-HCV chemical entities are evaluated in clinical trials, the prediction of lifelong clearance of HCV infection is often assessed by a surrogate endpoint termed sustained virologic response (SVR). SVR is determined at ≥ 12 weeks after discontinuation of antiviral therapy and defined as non-detectable HCV RNA in serum that is measured by sensitive assays such as a polymerase chain reaction (PCR) based determination. Two earlier predictors of SVR during clinical trials are rapid virologic response (RVR) and early virologic response (EVR) which are defined as reduction of serum HCV RNA to PCR-non-detectable levels by week 4 and achievement of ≥ 2 $\log_{10}$ reduction in serum HCV RNA level by week 12, respectively. Not all persons who eventually achieve an SVR will have met the EVR or RVR milestones and conversely reaching any or all of these three categories do not guarantee lifelong clearance of detectable HCV infection. However, these guidelines are predictive of ultimate therapy success and help clinicians determine, for example, if persons with significant side effects that fail to achieve EVR or RVR should discontinue treatment.

Nucleoside analogs that target the HCV NS5B polymerase that have reached human clinical trials will be the focus of this review as they have demonstrated significant advantages in the clinic with broader activity against the various HCV GT and a higher barrier to the development of resistant viruses when compared to all other classes of HCV inhibitors (McCown et al., 2008). The HCV NS5B polymerase catalytic site is highly conserved across the various genotypes resulting in little variation of potency of the nucleoside triphosphate inhibitors that target this protein (Asselah and Marcellin, 2012; Waheed et al., 013). Selection of HCV mutants *in vitro* has been quite difficult with most nucleoside inhibitors and selected mutants have been found markedly unfit with little to no clinical significance (Najera, 2013; Tong et al., 2013). There have been several nucleoside analogs discovered that exhibit selective anti-HCV activity, most with modifications at the 2′ and/or 4′ positions of the ribose sugar. However, nucleoside analogs as a class have potential problems such as poor passive cellular permeability and the modified nucleoside may not be a suitable substrate for the three successive cellular kinases that are necessary to transform it to the biologically active nucleoside analog triphosphate (NTP) (Fig. 2). Since HCV lacks nucleoside kinase expression, conversion to the biologically active nucleoside analog triphosphate relies on the human cellular kinases (Meppen et al., 2009). In addition, antiviral activity of nucleoside analogs is heavily influenced by the levels of natural dNTP or rNTP (Fig. 2) since they compete for incorporation by viral polymerases. Nucleoside analog inhibitors of viral RNA polymerization have the additional challenge of 10 to 100 times higher levels of rNTPs relative to the corresponding dNTP (Kennedy et al., 2010; Medivir, 2011a, b; Traut, 1994). Additionally, another significant obstacle in the development of nucleoside analogs for HCV therapy is their potential to be substrates and/or inhibitors of host polymerases. As an example, in the recent unfortunate events related to the 2′-methyl substituted guanosine analogs **IDX184** and **INX-189**, the human mitochondrial RNA polymerase has become of significant concern (Arnold et al., 2012).

Prodrugs are defined as a pharmacological substance administered in an inactive (or significantly less active) form that is metabolized into an active metabolite. Prodrugs are most often employed to improve absorption, distribution, metabolism, and/or elimination (ADME) properties of the parent pharmacological substance. One of the most common applications of prodrugs is for a drug that is poorly absorbed through the gastrointestinal tract to improve oral bioavailability. A prodrug may also be used to improve the selectively by which the drug interacts with the intended target cells or processes versus those that are

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not the drug's intended target. In the HCV area, nucleoside prodrugs have demonstrated both improvement of oral bioavailability and the ability to selectively deliver nucleotide analogs to liver tissue by first pass metabolism. Nucleoside analogs themselves are prodrugs of the active triphosphate form, which is recognized and incorporated by viral polymerases or reverse transcriptases. Antiviral activity of a nucleoside analog can often be improved by conversion to a prodrug as this aids in absorption and/or cellular penetration. In the case of monophosphate prodrugs, the potential rate limiting initial phosphorylation step is bypassed leading to higher intracellular nucleoside analog monophosphate levels which in many cases leads to significantly higher triphosphate levels that can translate to a significant enhancement in antiviral activity (De Clercq and Field, 2006; Hecker and Erion, 2008; Li et al., 2008; Poijärvi-Virta and Lönnberg, 2006; Sofia, 2011; Sofia et al., 2010a).

Prodrugs have played a central role in the delivery of active agents to their target tissue with improved efficiency and fewer side effects for well over 100 years. One of the earliest and enduring prodrug applications was to salicylic acid which when converted to acylsalicylic acid (Gerhardt, 1853; Von Gilm, 1859), significantly reduced irritation to the mouth and gastrointestinal tract (Fig. 3). Salicylic acid has been used for thousands of years to ease pain and reduce fever, but it was not until the period from 1826 to 1838 that salicylic acid was isolated by a series of chemists from willow tree bark (Jeffreys, 2005).

In 1856 an industrial scale process to make salicylic acid was developed from phenol and carbon dioxide (Kolbe, 1860; Schmitt, 1885), but interestingly the correct chemical structure was not assigned until 1869 that the correct chemical structure was assigned (Schröder and Kraut, 1869). In 1897, approximately 44 years after the first synthesis, acylsalicylic acid was first prepared by Bayer and in less than two years aspirin was trademarked and was being sold worldwide. As of 2013 (160 years after the first synthesis) more than 41 kilotons (metric) of aspirin is consumed annually worldwide. Many modern prodrugs utilize the same simple ester masking of a free hydroxyl group as a prodrug strategy as is exemplified by 3′-L-valinyl ester prodrug **NM283** (Fig. 4) or 2′,3′,5′-triisobutyrate ester prodrug **R1626** (Fig. 5) and 3′,5′-diisobutyryl ester prodrug **RG7128** (Scheme 4) which are among the first HCV clinical candidates.

## 2. Valopicitabine (NM283): the first clinical HCV nucleoside

### 2.1. Preclinical development

2′-*C*-Methylcytidine (Harry-O'kuru et al., 1997) also known as **NM107** (Sommadossi et al., 2008; Sommadossi and La Colla, 2001a, b; Sommadossi et al., 2004a; Sommadossi et al., 2004b, c; Storer et al., 2004), was discovered by Idenix Pharmaceuticals to be a potent inhibitor of RNA viruses replication such as bovine viral diarrhea virus (BVDV), West Nile virus (WNV), dengue-2 virus, yellow fever virus (YFV) and subsequently HCV (Kowdley et al., 2013; Sommadossi and La Colla, 2001b). Despite its broad spectrum *in vitro* activity, **NM107** displayed low oral bioavailability. To overcome this issue, **NM283** (Sommadossi et al., 2008; Sommadossi and La Colla, 2001a, b; Sommadossi et al., 2004a; Sommadossi et al., 2004b, c; Storer et al., 2004) also called valopicitabine, the 3′-*O*-L-valinyl ester prodrug of **NM107**, was developed (Pierra et al., 2005). In March 2006, Novartis announced to exercise its option from Idenix Pharmaceuticals to in-license the late stage development of valopicitabine.

### 2.2. Synthesis of valopicitabine (NM283)

Large scale synthesis of **NM107** and **NM283** were achieved from commercially available D-fructose by first reaction with calcium oxide and then treatment with oxalic acid to give the key 2′-*C*-Me-lactone **1**. Subsequent benzoylation, reduction of the lactone with Red-Al

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and benzoylation of the lactol **3** afforded compound **4**. At this stage, Vorbrüggen type coupling with cytosine using $SnCl_4$ as Lewis acid followed by debenzoylation gives access to **NM107. NM283** is finally obtained from **NM107** after simple peptidic coupling with *N*-Boc-L-valine and Boc-removal under acidic condition (Scheme 1) (Pierra et al., 2005).

## 2.3. Clinical development

**2.3.1. Phase I/II**—In 2004, the first phase I/II study of valopicitabine was reported, wherein 48 subjects received valopicitabine at 50, 100, 200, 400 and 800 mg/day orally once daily for 15 days with 14 days of follow-up; a subset of subjects received 400 mg twice daily (Godofsky et al., 2004). During these escalation trials, a dose-related decrease of serum HCV RNA at Day 16 was observed ranging from 0.15 $\log_{10}$ at the lowest dose to 0.73 $\log_{10}$ at 400 mg/twice daily. At 400 mg/day, 50% of the subjects experienced transient mild nausea and 20% had vomiting, but all completed treatment without modifications.

**2.3.2. Phase IIa**—Following the discovery of a synergistic antiviral effect against BVDV, *in vivo*, between **NM107**, the parent compound of valopicitabine (**NM283**) and **IFN**, a combination study with treatment-naïve subjects involving valopicitabine and **IFN** was initiated (Afdahl et al., 2005). Valopicitabine was given for 24 weeks with escalation to 800 mg/day over the first week before **IFN** was administered (1.0 μg/kg weekly) on Day 8. Along with a satisfactory tolerance, HCV RNA reductions increased to 3.2 $\log_{10}$ IU/mL for the combination treatment group (compared to 1.0 $\log_{10}$ IU/mL for monotherapy group). Eleven of twelve subjects treated for at least 10 weeks had substantial HCV RNA reductions superior to 2 $\log_{10}$ and four subjects were PCR-negative (<10 IU/mL).

**2.3.3. Phase IIb**—In a Phase IIb clinical trial, a 48 weeks combination of valopicitabine and **IFN** was evaluated in treatment-naïve subjects with HCV GT1 (Dieterich et al., 2006; Lawitz et al., 2007). This study was divided into five treatment groups: (A) **IFN** alone (180 μg QW = once weekly) for 4 weeks, valopicitabine 800 mg/day starting at week 5; (B) valopicitabine 200 mg/day + **IFN** (180 μg QW); (C–E) valopicitabine 800 mg/day with various week 1 induction regimens + **IFN** (180 μg QW). However, due to high gastrointestinal (GI) side effects at 800 mg/day dose of valopicitabine, the protocol was amended to reduce the dose to 200 or 400 mg/day after 14–22 weeks of treatment. The rate of viral decline was shown to be valopicitabine dose-related through week 4. HCV RNA was below 600 IU/mL in most subjects by week 12 and below 20 IU/mL by week 24 and 36. Group B maintained suppression of HCV RNA through week 48 along with satisfactory tolerability.

**2.3.4. Phase IIb**—In another Phase IIb trial (Afdahl et al., 2005), a 72 week three arm combination study involving valopicitabine/**IFN** in non-responder subjects was designed. Subjects were randomized to: (A) valopicitabine monotherapy (800 mg/day) and 3 combinations arms: **IFN** (180 μg SQ/week) with **RBV** (1,000–1,200 mg/day) + (A) valopicitabine (400 mg/day); (C) dose escalation 400–800 mg/day; (D) 800 mg/day or **IFN**/**RBV** retreatment. Again, due to GI issues with the 800 mg/day dose, the maximum dose was reduced to 400 mg/day. At week 48, HCV RNA suppression of treated persons with doses of 800 mg/day or escalated-dose 400 to 800 mg/day of valopicitabine, was 6 fold greater (0.8 $\log_{10}$) than **IFN/RBV** treatment demonstrating the potency of valopicitabine for the treatment of non-responder persons.

## 2.4. Conclusions

Twelve days after Idenix announced on July 1st 2007, that a triple therapy with valopicitabine and **IFN/RBV** cleared HCV RNA in 72% of subjects after 12 weeks of

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treatment, the FDA requested that the clinical trials on valopicitabine be discontinued due "to the overall risk/benefit profile observed to date in clinical testing" (Idenix, 2007). From the beginning, this drug as plagued with GI issues and unfortunately was not potent enough to be used alone or even when combined with the standard of care (**IFN/RBV**).

## 3. R1479 and its prodrug R1626: balapiravir

### 3.1. Preclinical development

Discovered and developed by Hoffmann-La Roche, Inc., but aided by technology and compound libraries obtained by the 1994 purchase of Syntex Research, the 4′-azidocytidine derivative **R1479** (Ahmed et al., 2007; Connolly et al., 2005; Devos et al., 2002; Harrington et al., 2008; Martin et al., 2004) was identified as a potent and selective inhibitor of HCV RNA polymerase. **R1479** displayed an $EC_{50}$ of 1.3 μM in an HCV replicon system and did not exhibit cytotoxic or cytostatic behavior in the Huh-7 replicon cell line (Smith et al., 2007). The triphosphate form of **R1479** was found to be a substrate for HCV polymerase and it inhibited RNA chain extension after incorporation as a non-obligatory chain terminator with an $IC_{50}$ of 320 nM (Klumpp et al., 2006). *In vitro* and *in vivo* assessment of **R1479** revealed that like many other nucleoside analogs, **R1479** was a polar molecule with a low aqueous solubility of 10 mg/mL that led to limited absorption after oral administration (Alfredson et al., 2006; Toniutto et al., 2008). Further, **R1479** exhibited low Caco-2 permeability [(Artursson et al., 1996); Caco-2 cells are used as a model system to predict intestinal epithelial permeability] and oral bioavailability values ranging from 22% in monkey to 89% in rat. Oral absorption in human clinical studies showed 6–18% based on urine recovery.

Consequently, prodrug approaches were investigated (Alfredson et al., 2006) to improve the oral bioavailability of **R1479**. Prodrug approaches that increase lipophilicity of a compound increase the potential for passive transport across cellular membranes and thus potentially improve absorption and transport across cellular membranes. The first approach focused on using esters and amides at the 2′, 3′ or 5′ positions of sugar to increase the lipophilicity, the second approach focused on increased lipophilicity with liver targeted cleavage using carbamate prodrugs at N4 position of cytosine and the third approach focused on amino acid esters to potentially target the PepT1 transporter for increased active intracellular transport. A five-fold increase in plasma exposure was observed with tri-isobutyrate ester prodrug **R1626** (Ahmed et al., 2007; Connolly et al., 2005; Devos et al., 2002; Harrington et al., 2008; Martin et al., 2004) in oral bioavailability in rats and monkeys. Further, results from Caco-2 experiments (Brandl et al., 2008) indicated that the hydrolysis of the prodrug is rapid and converted to **R1479** by esterases in gastrointestinal epithelial cells. Exceptional enhancement of dose proportionality combined with a 3-fold enhancement of plasma exposure both in human studies versus oral administration of **R1479** resulted in the selection of tri-isobutyrate ester prodrug **R1626** for further clinical evaluation.

### 3.2. Synthesis of R1479 and its prodrug balapiravir (R1626)

The synthesis of 4′-azidocytidine (**R1479**) (Connolly et al., 2005) and its triisobutyrate ester prodrug **R1626** (Sarma, 2007) is outlined in Scheme 2. Starting from uridine, 5′-iodo uridine was prepared then base eliminated to the 4′-exocyclic methylene nucleoside analog **7**. Addition of iodoazide across the double bond and benzoylation gave the 5′-iodo,4′-azido bis-benzoylated nucleoside **8**. Oxidative displacement of the 5′-iodo gave the 5′-*m*-chlorobenzyl ester **9** that was converted to the cytidine analog and debenzoylated to give **R1479. R1626** was then formed in good yield by reaction with isobutyric anhydride in the presence of potassium hydroxide (Scheme 2).

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### 3.3. Clinical development

**3.3.1. Phase I**—Starting in 2006, balapiravir (**R1626**) was given to healthy subjects in single doses from 500 mg to 12,000 mg to evaluate the safety, tolerability and pharmacokinetics. High levels of **R1479** was found in plasma accompanied by low levels of both mono and diester analogs. **R1479** was found in plasma in a linear amount proportional to all doses studied. Forty healthy male subjects (13 to 34 years old) were randomized 3:1 for treatment with **R1626** or placebo and were followed up for 7 days. The most common reported adverse effects were headache and irritation at the site of ECG electrode application. The phase I study concluded that balapiravir was efficiently absorbed, rapidly converted to **R1479** and was tolerated up to a dose of 12,000 mg when orally administered in healthy subjects (Robson et al., 2007).

**3.3.2. Phase Ib**—This clinical trial was designed to study the effect of **R1626** administered as monotherapy in HCV-infected persons. A total of fourty-seven GT1 subjects were randomized to oral treatment of **R1626** with doses of 500, 1,500, 3,000, 4,500 mg and placebo for 14 days (Roberts et al., 2008). As seen in the phase I study, after oral administration, **R1626** was efficiently converted to **R1479** with dose proportional pharmacokinetics observed over the entire dose range. The mean viral concentration at baseline was 6.3 $\log_{10}$ IU/mL, after 2 weeks of dosing; the mean (and median) viral concentration decreased by 1.2 (0.8), 2.6 (2.7) and 3.7 (4.1) $\log_{10}$ at 1,500, 3,000 and 4,500 mg, respectively. **R1626** was generally well tolerated up to 3,000 mg twice a day with mild side effects and no serious adverse events. However, there were increased adverse events at the highest dose of 4,500 mg BID. The main adverse effect seen was reversible, mild to moderate hematological changes. This study resulted in a maximum dose of 3,000 mg twice a day for **R1626** in all future clinical trials. The study concluded that treatment with **R1626** as monotherapy significantly reduced plasma HCV viral load.

**3.3.3. Phase IIa**—In this study drug resistance, efficacy and safety of the combination therapy of **R1626** along with **IFN** with and without **RBV** was studied over four weeks in 107 GT1 treatment-naïve patents. Subjects were randomized to 1,500 mg or 3,000 mg BID of **R1626** with **IFN** or 1,500 mg BID with **RBV**. Control group received standard of care (SOC) as **IFN** and **RBV** (Pockros et al., 2008). After the four-week treatment period all four arms continued on SOC out to 48 weeks. Results were promising at week 4 with undetectable HCV RNA (<15 IU/ml) in 29%, 69% and 74% of subjects receiving 1,500 mg BID dual therapy, 3,000 mg BID dual therapy, and 1,500 mg BID triple drug therapy, respectively, compared with 5% in the SOC arm. Sequence analysis of the entire NS5B coding region of the samples from these subjects revealed no known **R1479** resistance mutations (S96T or S96T/N142T) when compared with the baseline sequence. The main adverse event noted was grade 4 neutropenia that occurred in 50% of the persons in the **R1626** treatment groups and occurred with 78% of the persons in the 3,000 mg BID group compared to 10% in the SOC arm. Overall, this study had safety concerns related to the drug; however, most AEs were mild (80%) or moderate (16%), but 4% were rated as severe. Strong synergistic antiviral effect was seen with **R1626**, **IFN** and **RBV**, which suggested that the dose of one or more of these drugs could be lowered to improve the tolerability without significantly compromising efficacy.

**3.3.4. Phase IIb**—This double-blind phase II trial assessed the optimal treatment protocol for balapiravir plus **IFN/RBV** (Nelson et al., 2012). Seven arms of treatment-naïve GT1 infected subjects (N = 516) were randomized and received balapiravir 500, 1,000, or 1,500 mg twice daily, **IFN** 180 or 90 μg/week and **RBV** 1,000/1,200 mg/day or **IFN/RBV**. Safety concerns reduced the planned treatment duration with balapiravir from 24 to 12 weeks. From week 2 to week 12 undetectable HCV RNA was more common in all balapiravir

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groups. Ultimately, the efficacy assessment was compromised by high rates of dose modifications and discontinuations, which resulted in similar SVR rates in the balapiravir groups (32–50%) and **IFN/RBV** group (43%). Balapiravir showed dose related serious adverse events (especially hematological, infection, ocular events) and discontinued for safety reasons in 28–36% of patients (most often for lymphopenia). Balapiravir was considered possibly related to two deaths in the balapiravir/**IFN/RBV** combination groups.

### 3.4. Conclusions

In Oct-2008, following an unacceptable benefit to risk ratio revealed in later Phase IIb study and lack of superior efficacy, **R1626** was withdrawn from further consideration of future drug development in the treatment of chronic hepatitis C (Klumpp and Smith, 2011; Roche, 2008). Interestingly, **R1626** was evaluated in adult patients infected with dengue virus in a double-blind placebo controlled trial at 1,500 and 3,000 mg BID for five days. In August 2012, Roche reported **R1626** to be well tolerated but it demonstrated no viremia improvement versus the placebo arm (Nguyen et al., 2013). In our hands the parent 4′-azidocytidine nucleoside was a weak inhibitor in the HCV replicon system ($EC_{50}$ = 11 μM and $EC_{90}$ = 32 μM) and it is unclear why this drug was selected to be developed in humans.

## 4. RG7348 (MB11362)

A collaboration and license agreement between Metabasis Therapeutics, Inc. and Roche began in 2008 for the development of new treatments for HCV infection utilizing pre-clinical candidate **MB11362** and the proprietary HepDirect® liver-targeting technology. The lead HepDirect nucleoside, renamed **RG7348**, was declared a clinical candidate in the second quarter of 2009. In January 2010, Ligand acquired Metabasis and subsequently, Roche advanced **RG7348** into Phase I clinical studies (Ligand, 2010).

### 4.1. Preclinical development

The structure of **RG7348** has not yet been disclosed, but it is known that it is a double prodrug that acts as a potent and selective HCV NS5B polymerase inhibitor (Nieforth et al., 2012). **RG7348** was designed to provide high intra-hepatic levels of 4′-azidouridine triphosphate (Fig. 6). Very little preclinical data has been disclosed for **RG7348**. After oral dosing, the double prodrug was subsequently transformed by esterases and CYP3A4 (cytochrome P450 3A4) to 4′-azidouridine monophosphate **10**, which was converted by kinases to the active 4′-azidouridine triphosphate **11**, a potent and selective inhibitor of HCV polymerase.

### 4.2. Clinical development

**4.2.1. Phase I—**In 2012 Nieforth and co-workers reported a Phase I study on safety, pharmacokinetics (PK) and anti-HCV activity for GT1 subjects with **RG7348**. The study was a randomized, double-blind, placebo-controlled single ascending dose study in healthy subjects (part A: 60 subjects with 12/placebo and 8/cohort receiving 15 mg, 50 mg, 150 mg, 300 mg, 600 mg and 1,200 mg), and multiple dose study in chronic HCV GT1 subjects (part B: 12 male subjects: 4/placebo and 8/**RG7348** 150 mg BID). After **RG7348** dosing, $C_{max}$ and $AUC_{inf}$ for **(10)** and **(11)** increased dose proportionally from 15 to 150 mg and less than dose proportionally at higher doses. **RG7348** was well tolerated in chronic HCV persons in this proof-of-concept study. Although **RG7348** was a novel liver-targeted HCV nucleotide polymerase inhibitor, which demonstrated promising safety and PK, there was limited observed antiviral effect (mean reduction in HCV RNA <0.8 $\log_{10}$ IU/mL at all time points) at the dose studied.

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### 4.3. Conclusions

In November 2010, Roche terminated the HCV development with Ligand. There was no statement from either company that explained the basis of this decision. In January 2011, Ligand Pharmaceuticals Inc. announced a strategic relationship with Chiva Pharmaceuticals to develop multiple Ligand assets. Chiva was granted a non-exclusive HepDirect® technology license for the discovery, development and commercialization of new compounds for HCV treatment, including **RG7348**. No other advances for **RG7348** have been reported to date. Although the HepDirect technology seemed interesting, it never delivered on the promise to be liver specific and to add value to the nucleoside analogs studied.

## 5. PSI-6130

### 5.1. Chemical synthesis

β-D-2′-Deoxy-2′-fluoro-2′-*C*-methyl-cytidine (**PSI-6130**) was conceived from a combination of 2′-methyl of the HCV active **NM107** with the non-selective 2′-deoxy-2′-fluorocytidine (Cedilote et al., 2008; Clark, 2005). Also, at about the same time, Pharmasset scientists discovered the modest anti-HCV activity of gemcitabine which was another 2′-disubstitued nucleoside (Stuyver, 2003). **PSI-6130** was first synthesized from commercial available cytidine in about 3% total yield (Clark et al., 2005), but a more efficient, diasteroselective and scalable process was developed using commercially available isopropylidene protected D-glyceraldehyde (Wang et al., 2009). Thus, Wittig type reaction was performed with aldehyde **12** to give the unsaturated derivative **13** (Scheme 3). After oxidative dihydroxylation, compound **15** was selectively fluorinated. Cyclisation of ester **16** under acidic conditions gave lactone **17,** which after benzoylation, reduction of the lactone and further acetylation gave the key sugar **19**. Finally, introduction of the cytosine base under Vorbrüggen type coupling condition and complete debenzoylation afforded **PSI-6130** in a 6.4% overall yield.

### 5.2. Preclinical development

**PSI-6130** is a potent and specific HCV RNA-dependent RNA polymerase (RdRp) inhibitor with $EC_{90}$ of 5.4 ± 2.6 μM in Huh-7 replicon cells (Clark et al., 2005; Stuyver et al., 2006). It is interesting to note that just a few years earlier the commonly used BVDV surrogate assay would not have identified the anti-HCV activity of **PSI-6130** because **PSI-6130** was found to be highly specific for HCV over other RNA or DNA viruses. The 5′-triphosphate of **PSI-6130** was found to be equally active against the wild-type GT1b replicon clone and the S282T mutant replicon (Murakami et al., 2007). **PSI-6130** showed no apparent cytotoxicity against various cell types, including human bone marrow and human peripheral blood mononuclear cells. No mitochondrial toxicity was observed with **PSI-6130** (Stuyver et al., 2006). An *in vitro* triple combination study of **PSI-6130** with **IFN** and **RBV** at 1:1:1 ratio indicated synergistic antiviral effect, with no apparent cytotoxicity effects with any of the combinations tested (Bassit et al., 2008). Cell-based metabolism studies with primary human hepatocytes indicated that **PSI-6130** is a unique compound that gives rise to two nucleoside triphosphates that are both potent inhibitors of the HCV RdRp (Fig. 7) (Ma et al., 2007; Murakami et al., 2007; Murakami et al., 2008). **PSI-6130** can be converted to an inactive uridine analog **PSI-6206** (also known as **RO2433**) by human cytidine deaminase (CDA) and to **PSI-6130**-MP by human 2′-deoxycytidine kinase (dCK), respectively. The formed **PSI-6130**-MP produced two active nucleoside triphosphates through two separate pathways. Thus, phosphorylation of **PSI-6130**-MP to the 5′-di- and 5′-triphosphates was catalyzed by uridine-cytidine monophosphate kinase (YMPK) and nucleoside diphosphate kinase (NDPK), respectively. In addition, metabolism studies with **PSI-6130** showed that the

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monophosphate of **PSI-6130** was deaminated by 2′-deoxycytidylate deaminase (DCTD) to the uridine monophosphate derivative by and subsequently anabolized to the 5′-triphosphate **RO2433**-TP by YMPK and NDPK in a second pathway (Fig. 7). The inactive uridine analog **PSI-6206** is not a substrate for dCK and cannot be converted to **PSI-6206**-MP directly. The first cellular activation step to monophosphate is the rate-limited step among the three phosphorylation steps. Interestingly, the monophosphate species of **PSI-6130** and **PSI-6206** are very rapidly transformed into their corresponding nucleoside triphosphates, since those compounds are found at the highest concentration among all phosphate species present. Finally, **PSI-6130**-TP was a more potent inhibitor of the wild-type and S282T mutant polymerase compared to **PSI-6206**-TP (Ali et al., 2008; Ma et al., 2007; Murakami et al., 2007). Notably, **PSI-6206**-TP had a significantly longer half-life than **PSI-6130**-TP (see below).

### 5.3. Clinical development

Early animal studies involving single-dose pharmacokinetics studies with **PSI-6130** in rhesus monkeys showed low oral bioavailability for **PSI-6130** (Asif et al., 2007). An early phase I study in humans found a significant conversion to the inactive uridine analog combined with a bioavailability of 25%.

### 5.4. Conclusions

Because of these factors, a prodrug strategy was explored to potentially enhance bioavailability and provide a more selective conversion to **PSI-6130**-TP. **RG7128**, a prodrug of **PSI-6130** (refer to Section 6 of this manuscript), was ultimately selected for clinical evaluation based on its improvements in bioavailability and pharmacokinetics.

## 6. RG7128 (R7128, RO5024048, mericitabine)

### 6.1. Preclinical development and chemical synthesis

**RG7128** (mericitabine) is a 3′,5′-diisobutyryl ester prodrug and was prepared directly from **PSI-6130** with isobutyryl chloride in the presence of DMAP and TEA in THF/$H_2O$ at 0 °C (Scheme 4) (Chun et al., 2007).

Mericitabine has excellent oral bioavailability but has very little plasma exposure and is quickly converted to **PSI-6130** in humans. Metabolism of **PSI-6130** proceeds by a bifurcated pathway to more potent triphosphate species **PSI-6130**-TP and less active uridine analog **PSI-6206**-TP and both serve as non-obligate chain terminators (Ali et al., 2008; Ma et al., 2007; Murakami et al., 2007; Murakami et al., 2008). The prodrug mericitabine demonstrated potent inhibition of stable and transient replicons with $EC_{50}$ values similar to those of **PSI-6130**. Both mericitabine and **PSI-6130** inhibited HCV GT1a (H77 strain) and GT1b (Con1 strain) subgenomic RNA replication, with similar levels of potency (mean $EC_{50}$ values, 0.31 μM and 0.51 μM, respectively) (Ali et al., 2008).

### 6.2. Clinical development

**6.2.1. Phase I**—In a two week phase I monotherapy clinical study with 32 HCV GT1 patients, mericitabine showed a mean 2.7 $\log_{10}$ reduction in viral RNA with a dose of 1,500 mg BID (Reddy et al., 2007). HCV RNA levels were unchanged at Day 15 in six subjects who were given oral placebo in this study. In a four week, randomized, placebo-controlled phase I trial, 88% and 85% of treatment-naïve subjects, who were given 1,000 mg or 1,500 mg BID of mericitabine in combination with SOC (**IFN/RBV**), achieved undetectable RNA levels, respectively (Rodriguez-Torres et al., 2008). By contrast, only 19% subjects in SOC control group achieved no virus detectable level.

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**6.2.2. Phase IIb**—In one of the phase IIb clinical trials (PROPEL) (Jensen et al., 2010; Wedemeyer et al., 2013), 408 GT1 and GT4 treatment-naïve subjects were randomized into one of five treatment arms: arm A: mericitabine 500 mg BID for 12 weeks; arm B: mericitabine 1,000 mg BID for 8 weeks; arm C: mericitabine 1,000 mg BID for 12 weeks; arm D: mericitabine 1,000 mg BID for 12 weeks; and arm E: placebo BID for 12 weeks. All subjects received **IFN/RBV** at standard doses for 24 or 48 weeks during and after mericitabine/placebo treatment. Subjects in arm A, B and C who achieved extended rapid virologic response (eRVR) stopped all treatment at week 24. Subjects who did not have an eRVR, and persons in arm D and E continued SOC to complete the 48 weeks treatment. Overall, the virologic response rates were higher in arms A–D than in arm E at weeks 4 and 12. However, the arms D (50.6%) and E (placebo, 51.2%) had similar overall SVR24 rates (primary outcome) with lower rates in the response-guided therapy arms A, B and C (48.8%, 42.0% and 32.9%, respectively).

These data demonstrated that treatment with mericitabine plus **IFN/RBV** for 8 or 12 weeks provided potent suppression of HCV RNA, was well tolerated, did not result in selection of resistant variants; however, it did not increase SVR rates compared with placebo.

An analysis from the now completed phase IIb JUMP-C trial was reported by Pockros and colleagues (Pockros et al., 2013). A total of 166 treatment-naïve persons infected with HCV GT1 or GT4 were randomized to one of two treatment arms. Subjects randomized to arm A were given mericitabine BID plus SOC (**IFN/RBV**) with response guided therapy (RGT). Persons on arm A who achieved eRVR (extended rapid virologic response; means no virus detected at week 4 and week 12) stopped all treatment at week 24. Those persons who did not achieve an eRVR also stopped the mericitabine at week 24, but continued an additional 24 weeks of SOC treatment. The subjects in control group arm B received SOC treatment for 48 weeks. The results of JUMP-C phase IIb trials showed that mericitabine was safe, and associated with higher SVR rates when compared to SOC (56.8% vs. 36.5%). The virologic response obtained at weeks 4 and 12 in the JUMP-C trial was comparable to results observed with the PROPEL trials. However, the authors note that extending treatment period from 12 to 24 weeks in the PROPEL study was not as effective in increasing SVR rates as found in the JUMP-C study. The difference in SVR rates may have contributed to differences observed in viral breakthrough rates since the PROPEL study showed an almost two-fold increase in relapse as compared to JUMP-C results (Pockros et al., 2013).

It is worth noting that a combination of mericitabine plus SOC was also effective in treating HCV GT2/3 relapsers or non-responder subjects (Gane et al., 2010). Twenty persons with GT2/3 infections, who had not achieved SVR with previous treatments were given mericitabine 1,500 mg BID plus SOC for 4 weeks, followed by SOC alone for 22–24 weeks. Ninety percent (18/20) of persons achieved RVR and 65% (13/20) achieved SVR at 12 weeks. This trial demonstrated that mericitabine plus SOC provided an effective regimen for the treatment of HCV GT2 and GT3 infected persons who have failed previous treatments.

INFORM-SVR, an ongoing phase IIb clinical trial, aims to evaluate the safety and efficacy of an all-oral **IFN**-free treatment with mericitabine and ritonavir-boosted danoprevir (**RG7227**, protease inhibitor, Fig. 8), with or without **RBV** in GT1a/1b treatment-naïve subjects. Among the different arms of the study, a 15% virus breakthrough rate was reported for GT1a and 1b persons (Gane et al., 2012). Viral breakthrough was associated with virus harboring protease inhibitor resistance conferring mutations. Importantly, a transient S282T mutation, an NS5B polymerase mutation associated with mericitabine resistance, was selected in one person.

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Finally, ANNAPURNA, an ongoing phase IIb clinical trial is evaluating the combination of setrobuvir (**ANA598**, **RO5466731**, non-nucleoside polymerase inhibitor, Fig. 8), danoprevir (**RG7227**, **R05190591**, protease inhibitor, Fig. 8) and ribavirin with or without mericitabine in patients with CHC who are either treatment-naïve or have previously experienced a null response to interferon-based treatment (Fig. 8). With an expected completion date of December 2013, this two-part five arm study will be looking at SVR12 and safety as end points. In part 2, based on results from part 1, further treatment-naïve subjects will receive a successful regimen from part 1, or a reduced intensity regimen, and interferon-based null-response persons will be added to the study (Roche, 2012).

### 6.3. Conclusions

The INFORM-SVR study is considered a milestone game changing combination since it demonstrated for the first time that an all-oral drug combination could suppress HCV replication to levels similar or exceeding SOC. It is also one of very few studies that show the selection of nucleoside analog associated resistance conferring mutations in humans, even though the apparently resistant virus was transient. The S282T mutation was not observed during other clinical triple combination trials with or without **IFN**, suggesting that the high barrier to resistance of mericitabine, and the low fitness of the mutant virus reduce the incidence of viral breakthrough caused by nucleoside analog resistance selection (Jensen et al., 2010; Le Pogam et al., 2010; Pockros et al., 2011; Pockros et al., 2013). The high rate of viral suppression with mericitabine combined with significant relapse rates without mutation selection indicates that longer treatments, higher doses, and/or other antiviral combinations may be necessary to make mericitabine a major player in HCV treatment. This may be due to the short half-life of **PSI-6130**-TP, **PSI-6130**-TP not reaching a viral reservoir(s), and/or overall lack of potency of **PSI-6130**-TP.

## 7. IDX184

### 7.1. Synthesis of IDX184

**IDX184** (Cretton-Scott et al., 2010; Sommadossi et al., 2008; Surleraux and Gosselin, 2011, 2012) was designed by Idenix Pharmaceuticals as a 2′-*C*-methyl guanosine (2′-*C*-MeG) ribonucleoside that contains a *S*-acyl-2-thioethyl (SATE) phosphoramidate prodrug to selectively deliver the 5′-MP of 2′-*C*-methylguanosine ribonucleoside to human liver. The metabolism of **IDX184** to 2′-MeG-MP takes place predominantly in liver cells and presumably involves cytochrome P450 (CYP450)-dependent and -independent processes (Zhou et al., 2011). By bypassing the rate-limiting monophosphorylation process, 2′-MeG-MP is then efficiently converted to its active 5′-TP within hepatocytes by cellular kinases. The complete metabolic pathway of **IDX184** is yet to be fully elucidated. Liver targeting may result in a favorable therapeutic index by increasing the levels of the active 2′-MeG-TP in liver cells while lowering systemic exposure to the nucleoside, potentially minimizing the risk of systemic side effects (Cretton-Scott et al., 2008).

**IDX184** was synthesized as shown in Scheme 5**Error! Reference source not found.** (Sommadossi et al., 2008). Preparation of the *H*-phosphonate monoester precursor was achieved in three steps from commercially available 2,2-dimethyl-3-hydroxypropanoic acid methyl ester **20**, by protection of the alcohol followed by saponification, leading to compound **21** in 92% yield without purification. Installation of the side chain was performed by peptidic coupling between compound **21** and 2-mercaptoethanol, to generate alcohol **22**. Finally, treatment of compound **22** with phosphorus acid and pivaloyl chloride, followed by quenching the reaction with triethylammonium bicarbonate (TEAB), generated *H*-phosphonate monoester precursor **23** in 90% over two steps.

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2′-*C*-Methylguanosine **24** and precursor **23** were then both treated with pivaloyl chloride to furnish intermediate **25**, which was further reacted with benzylamine to generate Tr-protected phosphoramidate diester **26** in quantitative yield. Classical deprotection with trifluoroacetic acid led to the isolation of **IDX184** in 39% yield (Scheme 5).

### 7.2. Preclinical development

In preclinical studies using an HCV replicon assay, **IDX184** exhibited 10 times greater potency than nucleosides that were in clinical development at the time. In a seven-day preclinical toxicology study of **IDX184**, no toxicities, including gastrointestinal or hematological, were observed in monkeys at doses greater than or equal to 600 mg/kg/day. In HCV GT1 infected chimpanzees, once daily oral administration of 10 mg/kg of **IDX184** produced a rapid and potent inhibition of HCV replication with mean viral load reductions of 2.5–4.0 $\log_{10}$ after four days of dosing (Standring et al., 2008, 2009). Studies were also performed to characterize resistance mutations profiles of **IDX184**. The S282T mutation was identified in all tested cell lines and emerged around 25–77 days. Although the S282T mutant gave **IDX184** a 23-fold increase in $EC_{50}$, low (5%) replicon capacity of S282T replicons was also observed as previously reported by Ludmerer et al. (Ludmerer et al., 2005). The signature S282T mutant and other drug-related resistance mutants were only established *in vitro* for cell-based administration as ultimately there were no mutant resistance found in clinical trials and no cross-resistance between **IDX184** and other classes of HCV antiviral agents (McCarville et al., 2011; McCarville et al., 2010). **IDX184** demonstrated additive antiviral activity in the HCV replicon in combination with HCV protease inhibitors and **IFN** and synergistic activity in combination with **RBV. IDX184** also remained fully active *in vitro* against HCV containing known protease and non-nucleoside inhibitor drug resistance mutations (Zhou et al., 2011; Zhou et al., 2009). As a result of its favorable preclinical profile, **IDX184** was selected as Idenix's lead candidate in January 2008 and Phase I clinical trials were started in July of the same year.

### 7.3. Clinical development

**7.3.1. Phase Ia—**In July 2008, a randomized, placebo-controlled, dose-escalating study was designed to evaluate the safety and tolerability of **IDX184** after oral administration in healthy subjects. Doses escalating from 5 mg to 100 mg were administered to fourty-eight healthy subjects for three days, and PK data were collected (Idenix, 2008b). The most common adverse event was dizziness, 5.6% of occurrences for the **IDX184** treated group compared to 25% for the placebo cohort. Mild to moderate adverse events occurred and resolved by the end of the study. No severe adverse effects (SAE) or dose-limiting toxicity were observed. The level of **IDX184** and 2′-*C*-MeG in plasma remained low and dose-dependent, consistent with a liver-targeting approach. The short half-life of **IDX184** (around 1 h) in plasma suggested that **IDX184** was unlikely going to accumulate over time. In comparison, 2′-MeG in plasma has a long half-life with $t_{1/2}$ of approximate 18–42 h for doses above 25 mg, which reflected the long half-life of 2′-MeG-TP in the liver. The $C_{24h}$ of 2′-C-MeG is 0.58–2.88 ng/mL for dose higher than 25 mg of **IDX184**. Overall, **IDX184** was safe and well tolerated at a single dose up to 600 mg/kg/day. The favorable PK supported once-daily dose administration therapy in future studies involving HCV infected individuals.

**7.3.2. Phase Ib—**In December 2008, a double-blind, dose-escalating study (Phase Ib) began to evaluate the safety and antiviral activity of **IDX184** in fourty-one treatment-naïve subjects infected with GT1 chronic hepatitis C (Idenix, 2008a). These persons received 25 mg QD, 50 mg QD, 75 mg QD and 100 mg QD or placebo QD for 3 days. **IDX184** was safe and well tolerated: the occurrences of adverse events were following similar pattern as the placebo-treated group with the most frequently adverse events reported being headache and

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dizziness. The PK analysis data in the study was consistent with those of the previous healthy volunteers study. The mean HCV RNA reduction ($log_{10}$) ranged from 0.47 to 0.74 after treatment with 25 mg to 100 mg cohorts at day four. Significant HCV viral load decline occurred in persons receiving doses from 75 mg to 100 mg (Lalezari et al., 2012; Lalezari et al., 2009; Lalezari et al., 2010). This study helped to determine optimal doses that could be chosen for testing in later studies.

**7.3.3. Phase IIa**—In November 2009, a total of eighty subjects were enrolled to evaluate the short-term safety, the antiviral activity and PK profile of **IDX184** in combination with **IFN/RBV** in subjects with GT1 chronic hepatitis C infection. Over 14 days, individuals enrolled in this phase IIa, randomized, double-blind study (Idenix, 2009) were given sequential doses of 50 mg QD, 50 mg BID, 100 mg QD, 100 mg BID, 150 mg QD, 200 mg QD of **IDX184** in combination with **IFN/RBV** and **IFN/RBV** as the placebo cohort. Most adverse events were mild, and no serious adverse events were associated with **IDX184** plus **IFN/RBV** treatment. The pattern of side effects was similar between treatment of **IDX184** plus **IFN/RBV** and treatment of **IFN/RBV** alone. At day 15 of **IDX184** combination treatment, mean viral load reduction ($log_{10}$) was: 50 mg QD (−2.7), 50 mg BID (−4.0), 100 mg QD (−4.2), 100 mg BID (−4.1), 150 mg QD (−4.3), 200 mg QD (−3.7) of **IDX184** in combination with **IFN/RBV** and −1.5 for **IFN/RBV**. For cohorts given **IDX184** 50 mg QD, 100 mg BID and 200 mg QD, undetectable viral level (< 15 IU/mL) were found in 13–29% of the persons, whereas for 50 mg BID, 100 mg QD and 150 mg QD, 40–50% of the individuals achieved undetectable viral levels. Moreover, all **IDX184** treated cohorts led to a low level of mean alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels compared to **IFN/RBV** cohort. In addition, the mean plasma concentration of 2′-MeG, which was identified as predictor of viral load started to decline at Day 14 (Lalezari et al., 2013; Lalezari et al., 2010; Membreno and Lawitz, 2011).

**7.3.4. Phase I combination of IDX184 and IDX320**—In June 2010, a clinical drug-drug interaction study was initiated to evaluate the combination of **IDX320** (a macrocyclic HCV protease inhibitor, Fig. 9) and **IDX184** in healthy subjects (Idenix, 2010). This study was designed as a double blind, multiple-dose study of twenty subjects who were administered 100 mg/day of **IDX184** in combination with 400 mg of **IDX320**. The first cohort (8:2) was either treated with **IDX320/IDX184** matching placebo for seven days followed by seven additional days with **IDX320/IDX184**, or **IDX320** matching placebo/ **IDX184** matching placebo. The second cohort (8:2) was given **IDX184/IDX320** matching placebo for seven days followed by seven additional days **IDX184/IDX320** or **IDX184** matching placebo/**IDX320** matching placebo. This study was designed to evaluate safety and tolerability and to assess the potential for a pharmacokinetic drug-drug interaction between **IDX320** and **IDX184** when the two drugs were administered in combination in healthy subjects. In September 2010, the FDA placed a clinical hold on **IDX184** and **IDX320** programs based on three serious adverse events (elevated liver function tests) that occurred during this combination study in healthy volunteers. In February 2011, the FDA removed the full clinical hold on **IDX184** and placed it on partial clinical hold allowing Idenix to advance in the clinic under the partial clinical hold (Idenix, 2011a).

**7.3.5. Phase I**—In April 2011, an open-label study was initiated to evaluate the relative bioavailability of **IDX184** and any potential food effect in healthy male subjects (Idenix, 2011b). The purpose of this study was to compare the amount of drug in the blood after taking either a capsule or a tablet form of the drug while fasting. Additionally, the amount of drug present in the blood after eating a meal was investigated and the safety of the tablet form of **IDX184** was evaluated in healthy volunteers. Twelve subjects received 50 mg BID **IDX184** capsules without food on Day 1, 50 mg BID **IDX184** tablets without food on Day

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8, and 50 mg BID **IDX184** tablets with food on Day 15. Blood levels of **IDX184** were measured in each study participant to determine pharmacokinetic parameters including bioavailability of **IDX184**, AUC and $C_{max}$ of **IDX184** and 2′-C-MeG. Results of this study have not been disclosed at the time of this publication.

**7.3.6. Phase IIb—**In July 2011, Idenix initiated a double-blind study of sixty-seven randomized subjects to evaluate the safety and the antiviral activity of **IDX184** in combination with **IFN/RBV** at doses of 50 mg or 100 mg QD for 12 weeks. The common adverse events were fatigue, nausea and headache, which were generally mild to moderate. No treatment-related cardiac or renal serious adverse events were observed during the study and the occurrences of all adverse events were similar between **IDX184**-combination therapy and **IFN/RBV**-therapy. Echocardiograms and N-terminal pro B-type natriuretic peptide (NT-proBNP), a biomarker that aids in the detection of congestive heart failure and cardiac dysfunction, were added in this study to address potential cardiac concerns. Heart rate, AV conduction and cardiac depolarization remained normal. Several subjects in each group developed new T-wave inversions that were not judged to be clinically significant. The increases seen in NT-proBNP levels were greater than 200% of baseline, but reversible. In the 50 mg and 100 mg of **IDX184** in combination with **IFN/RBV** cohorts, 94% and 87% of subjects, respectively, achieved undetectable virus level at a median of 8 weeks of treatment. At week 12, a 76% and 82% rate of complete EVR was observed in the 50 mg and 100 mg treatment arms, respectively (Lawitz et al., 2012a). In January 2012, Idenix submitted interim phase IIb data to the FDA requesting for the removal of the partial clinical hold on **IDX184**. In February 2012, the partial clinical hold on **IDX184** was removed by the FDA allowing the initiation of dosing in the phase IIb study.

### 7.4. Conclusions

In August 2012, the FDA placed **IDX184** and **IDX19368** (another HCV nucleotide polymerase inhibitor for which Idenix had previously filed an IND, but had not initiated clinical studies) on partial clinical hold due to cardiac adverse events seen in the phase II clinical trial of **BMS-986094** (refer to section 15 of this manuscript). All three drugs are 2′-*C*-methyl guanosine nucleotide prodrugs. In December 2012, Idenix completed the submission of requested cardiac safety data for **IDX184** to the FDA. Idenix had found no evidence for severe cardiac effects in all of their clinical studies to date. In the meantime, in October 2012, Idenix started a three-year observational study to evaluate the durability of sustained viral response and the kinetics of antiviral-resistant HCV in subjects who participated in studies of Idenix anti-HCV direct acting antivirals, including **IDX184**. In February 2013, the FDA communicated that **IDX184** (and **IDX19368**) programs would remain on clinical hold, and, as a result, Idenix decided to terminate the development of these programs. A reason for FDA concern over **IDX184** and related compounds is the resultant NTP derived from **IDX184** is identical to the NTP derived from **BMS-986094**. Cardiac toxicity for **BMS-986094** was demonstrated to be dose-related and the postulated reason for the cardiac toxicity was mitochondrial toxicity. Of note, 2′-*C*-MeG-TP has been shown to be a substrate for incorporation by human mitochondrial RNA polymerase (Arnold et al., 2012). However, cell free assays can be misleading since the nucleoside may never be phosphorylated in mitochondria. Nevertheless, Idenix decided not to pursue this drug further since by then they had identified nucleoside analogs with superior virological and toxicological profiles.

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## 8. PSI-6206 to PSI-7851 to GS-7977 (PSI-7977; sofosbuvir)

### 8.1. Discovery and preclinical profiling

The presence of 2′-deoxy-2′-fluoro-2′-*C*-methyluridine **PSI-6206** metabolite along with its triphosphate **PSI-6206**-TP was recognized during metabolism studies on **PSI-6130** in cell culture and in monkeys (see discussion Section 5.2). These compounds were evaluated for their anti-HCV activity in replicon assay (Ma et al., 2007). While the uridine nucleoside **PSI-6206** was devoid of anti-HCV activity in culture, **PSI-6206**-TP demonstrated a potent and selective inhibition of HCV NS5B polymerase with mean $IC_{50}$ = 1.19 μM. Subsequent stability studies using primary human hepatocytes showed that **PSI-6206**-TP had significantly longer half life ($t_{1/2}$ = 38 h) than its analogous cytidine triphosphate **PSI-6310**-TP ($t_{1/2}$ = 6 h) (Murakami et al., 2008).

Cellular pharmacology with **PSI-6206** demonstrated that it was very poorly phosphorylated to the monophosphate nucleotide (**PSI-6206**-MP), which could explain the lack of anti-HCV activity. The phosphoramidate prodrug approach was taken to bypass the initial unproductive phosphorylation step (Murakami et al., 2010). After *in vitro* and *in vivo* profiling of multiple potential prodrugs, phosphoramidate prodrug **PSI-7851** was selected as the lead compound. **PSI-7851**, an equal mixture of two phosphorous diastereoisomers **PSI-7976** and **PSI-7977** (Berrey et al., 2013; Chun et al., 2010; Clark, 2005; Cleary et al., 2013; Ross et al., 2010; Sofia et al., 2008) was shown to be a potent and selective inhibitor of HCV replication ($EC_{50}$ = 0.52 μM) with no associated bone marrow, mitochondrial or other cellular toxicity. Its potential efficiency was further demonstrated by high levels of triphosphate (**PSI-6206**-TP) formation in primary human hepatocytes and in the livers of rats, dogs and monkeys when administered *in vivo* (Sofia et al., 2010a). *In vitro* studies, showed **PSI-7851** had a broad range of antiviral activity across different HCV GTs (Lam et al., 2012). Based on impressive preclinical data, **PSI-7851** was selected for future development and progressed into phase I human clinical trials.

### 8.2. Synthesis of PSI-7851 and PSI-7977 (GS-7977)

Subsequently, a method was developed to separate the two **PSI-7977** and **PSI-7976** diastereoisomers. The $S_p$ diastereoisomer **PSI-7977** ($EC_{90}$ = 0.42 μM) was shown to be more potent inhibitor than the $R_p$ diastereoisomer **PSI-7976** ($EC_{90}$ = 7.5 μM) in replicon assays (Sofia et al., 2010a). However, when both diastereoisomers were tested in primary human hepatocytes almost equal amounts of active triphosphate (**PSI-6206**-TP) was formed, suggesting both isomers may be equally active in the liver cells (Murakami et al., 2010). Enzymatic studies showed Cathepsin A (CatA) and carboxylesterase 1 (CES) could both catalyze the first step hydrolysis (of the carboxyl ester linkage between the carboxylic acid from amino acid moiety and the isopropyl alcohol) of phosphoramidate prodrugs **PSI-7977** and **PSI-7976. PSI-7977** was found to be a better substrate for CatA while **PSI-7976** was preferentially hydrolyzed by CES1 (Murakami et al., 2010). The apparent discrepancy between replicon data and primary human hepatocytes was related to the different expression of these enzymes in replicon cell lines and primary human hepatocytes (Murakami et al., 2010; Sofia et al., 2010a). Both isomers were devoid of cytotoxicity when tested against a broad range of cell lines. Mitochondrial toxicity was evaluated up to 100 μM in both CEM and HepG2 cells and no toxicity was observed for **PSI-7977** while **PSI-7976** showed $CC_{90}$ of 72 and 68.6 μM for inhibition of mtDNA and rDNA, respectively in HepG2 cells (Sofia et al., 2010a). Pure $S_p$ isomer **PSI-7977** was selected for future clinical studies due to its higher potency in replicon assay and better safety profile. To date, **PSI-7977** (now called sofosbuvir or **GS-7977**) is the only nucleotide analog HCV polymerase inhibitor that has completed phase III clinical trials and this drug is expected to receive FDA approval by the end of 2013.

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### 8.3. Synthesis of single isomer PSI-7977 (sofosbuvir, GS-7977)

The first generation synthesis of **PSI-7977** involved direct reaction of phosphoryl chloride reagent **28a** with 2′-deoxy-2′-fluoro-2′-*C*-methyluridine (**PSI-6206**) and separation of the two diastereoisomers by crystallization (Sofia et al., 2010a) (Scheme 6). Ultimately, with some modifications to the chemistry and workups during process development, the yield from **(28a)** to the mix of **PSI-7976** and **PSI-7977** (48:51) was improved to 45.7% from which the single isomer **PSI-7977** was isolated in 15% yield after crystallization (Ross et al., 2010).

This approach was further optimized by using *p*-nitrophenol as the leaving group of the phosphorylating agent as shown in **(28b)** (Scheme 6). In this case a 47% of a 3:1 mix of **PSI-7977** and **PSI-7976** were obtained. Separation of **(28b)** into its single diastereomers gave the desired chiral phosphorylating reagent **28c**, which upon reaction under basic conditions gave clean inversion at the phosphorous centers to give **PSI-7977** as the single desired $S_p$ diastereomer. Pentafluorophenyl was used in a similar manner with the phosphorylating reagent **28d** which allowed for a multigram synthesis of single $S_p$ isomer **PSI-7977** without the need for a chiral catalyst, chiral auxiliary or column purification (Ross et al., 2011; Ross et al., 2010).

### 8.4. Clinical trials for PSI-7977 (GS-7977; sofosbuvir)

**8.4.1. Phase I**—In March 2009, clinical trials for **PSI-7851** (also known as **GS-9851**; mixture of diastereoisomers **PSI-7976** and sofosbuvir) were completed in 42 healthy volunteers. The administration of single dosage of 25 mg up to 800 mg QD was shown to be generally safe and well tolerated with no adverse events when compared with the placebo arm and with a pharmacokinetic profile consistent with once-daily dosing (Denning et al., 2012). Phase Ia trials with **PSI-7851** also evaluated safety and tolerability for three day monotherapy at different doses (50 mg, 100 mg, 200 mg, 400 mg) in treatment-naïve subjects. Reductions in viral load were observed without any serious adverse events when compared to the placebo arm (Lawitz et al., 2013c). In fact, the 400 mg QD dose led to a decrease of 1.76 $\log_{10}$ IU/mL after three days of treatment compared to the related mericitabine, which after 14 days at 1,500 mg with QD dosing, gave a 1.48 $\log_{10}$ IU/mL drop.

**8.4.2. Phase IIa**—The safety, antiviral activity and tolerability of three different doses of sofosbuvir in combination with **IFN/RBV** were assessed in treatment-naïve persons with GT1 HCV infection. Sixty-four subjects were randomized (1:1:1:1) to receive one of three once-daily doses of sofosbuvir (100, 200 and 400 mg) or placebo plus **IFN/RBV** for 28 days, which was followed by 44 weeks treatment with **IFN/RBV** alone therapy. All who received sofosbuvir experienced rapid virologic response (RVR) of 88%, 94% and 93% for 100, 200, and 400 mg dose, respectively. In comparison, only 21% of the subjects achieved RVR in the control arm. No viral breakthrough was observed during 28 days treatment; however, eight individuals had relapsed during **IFN/RBV** treatment (four on sofosbuvir 100 mg; two on sofosbuvir 400 mg and two on placebo arm). SVR24 rates were higher for subjects in all arms with sofosbuvir treatment (56% for 100 mg dose, 83% for 200 mg dose, and 84% for 400 mg dose) than those in the **IFN/RBV** arm (43% achieved SVR after 24 weeks). The most frequent adverse effects during 28 days treatment were fatigue, nausea, chills, headache and joint pain. No serious adverse events or discontinuation was reported in first 28 days. Sofosbuvir 200 and 400 mg doses were selected for future studies (Rodriguez-Torres et al., 2013).

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**8.4.3. Phase IIb**—Several phase IIb clinical trials have addressed the safety and efficacy of sofosbuvir treatment in the treatment of HCV GTs. Results from four studies (PROTON, ELECTRON, QUANTUM and ATOMIC) are summarized below.

**8.4.3.1 PROTON:** In this double-blind, randomized study the efficacy of sofosbuvir in combination with **IFN** and **RBV** in treatment-naïve persons with HCV GT1, 2 and 3 was evaluated. A total of 121 treatment-naïve subjects with GT1 infection were randomly assigned to three different groups (2:2:1 ratio) to receive **IFN/RBV** with 200 mg sofosbuvir, 400 mg sofosbuvir, or placebo for 12 weeks followed by additional **IFN/RBV** treatment for 12 weeks or 36 weeks depending on the subject's response. Among GT1 persons, 98% of subjects on triple therapy achieved RVR compared to 19% in the placebo group. 88% of those on 200 mg dose of sofosbuvir and 91% of those on 400 mg sofosbuvir achieved SVR12 while less than 50% achieved SVR in placebo cohort. All IL28-BT/T GT persons included in the study (13/13) achieved SVR12 following triple therapy. Finally, combination therapy with sofosbuvir was even more effective for treatment-naïve persons with GT2/3 HCV infections. In this cohort, all 24 subjects who stayed on therapy achieved SVR12 (Gane et al., 2013b; Lawitz et al., 2013b; Pham et al., 2004; Radkowski et al., 2005).

**8.4.3.2. ELECTRON:** This study was designed to evaluate the efficacy of sofosbuvir with **RBV**, with or without **IFN** in treatment-naïve or treatment-experienced persons with GT1, 2, or 3 HCV infections. Sofosbuvir monotherapy, reductions in **RBV** dose, and changes in duration of therapy were also evaluated. One hundred and fifty five subjects with all three HCV GTs were divided into eleven treatment groups (Radkowski et al., 2005). In a randomized cohort, fourty treatment-naïve subjects with HCV GT2 and GT3 infections were divided into four arms. All groups received sofosbuvir (400 mg QD) + **RBV** BID for 12 weeks. Three of the arms also received **IFN** for 4, 8 and 12 weeks, respectively. SVR24 was achieved for all subjects enrolled in the four arms (cohorts 1 to 4) (Gane et al., 2013b; Gane et al., 2012). In an additional arm (cohort 5), wherein ten treatment-naïve persons with HCV GT2 or GT3 received sofosbuvir monotherapy for 12 weeks, SVR24 rates were 60%. A shortened 8-week triple therapy arm (cohort 6) was also evaluated in ten GT2 or GT3 subjects and SVR24 was achieved for all participating subjects. In cohort 7, twenty-five treatment-naïve subjects with GT2 or GT3 were treated with sofosbuvir plus **RBV** for 8 weeks. Full (100%) SVR was achieved in this cohort. Cohort 8 evaluated treatment of ten GT2/3 subjects with sofosbuvir + reduced dose **RBV** (800 mg) and resulted in a reduced SVR rate of ~60%. Twenty-five treatment-experienced subjects with GT2 and GT3 HCV infections were treated with sofosbuvir and **RBV** (cohort 9). All subjects in this arm achieved RVR while 68% attained SVR at 12 weeks. Finally, a 12-week **IFN**-free sofosbuvir plus **RBV** treatment was evaluated in ten GT1 null-responders (cohort 10) and in twenty-five GT1 treatment-naïve subjects (cohort 11). Treatment-naïve subjects with GT1 achieved SVR12 of 84% while only one out of ten null-responder subjects achieved SVR12 (Table 1).

In summary, 12-week treatment with sofosbuvir and **RBV** resulted in 100% SVR24 in treatment-naïve, GT2/3 subjects. This regimen was less optimal for treatment-experienced subjects with GT2/3 or null-responders with GT1 infections. It was concluded that duration of less than 12 weeks or reduced **RBV** dose might adversely impact treatment efficacy. Reduced dosage of **RBV** in treatment-naïve difficult GT1 subjects in 24 weeks treatment was less efficient (SVR4 56%) than full dose **RBV** (77%). Addition of sofosbuvir to **IFN** + **RBV** regimen considerably improved the response rate among all GTs and shortened the duration of therapy. **IFN**-free combination showed high percent of cure rate in untreated individuals, but was less efficient in treatment-experienced patients or null-responders.

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**8.4.3.3. ATOMIC:** This phase IIb clinical trial evaluated the combination of sofosbuvir with **IFN/RBV** for 12- or 24-weeks in treatment-naïve GT1, GT4, and GT6 persons (a total of 332 subjects were enrolled). Additionally, treatment-naïve persons with GT1 were treated with sofosbuvir in combination with **IFN/RBV** followed by 12 week treatment with sofosbuvir alone or in combination with **RBV**. The results from this study suggest that 12 week triple combination therapy was as effective as 24 week treatment for this group of subjects. Furthermore, it was found that sofosbuvir, with or without **RBV** after initial treatment, can be effective in achieving high SVR rate (Kowdley et al., 2013).

**8.4.3.4. Phase II sponsored by the National Institute of Allergy and Infectious Diseases (NIAID):** Initiated in 2011, NIH investigators recruited 60 GT1 HCV treatment-naïve subjects, into a two-part open label study (Gilead, 2011). As a proof of concept, in the first part, ten patients with mild to moderate liver fibrosis were treated with 400 mg/day of sofosbuvir and weight-based **RBV** for 24 weeks. All nine subjects who completed the 24-week course had an undetectable level of the virus 24-weeks post treatment and were deemed cured. Subsequently, in the second group of fifty subjects where thirteen suffered from serious liver damage, all took 400 mg/day sofosbuvir with the subjects being split between weight-based and low-dose (600 mg/day) **RBV**. SVR-24 rates of 68% were observed in the weight-based group and 48% in the low-dose group (Osinusi et al., 2013). Seven grade 3 adverse events were reported (anemia, neutropenia, nausea, hypophosphatemia, and cholelithiasis or pancreatitis), but none resulted in discontinuation of treatment. This NIH-sponsored study of Gilead's sofosbuvir combined with **RBV** demonstrated a safe and effective interferon-free approach for even some of the toughest HCV cases.

**8.4.4. Phase III—**Multiple phase III clinical trials have been initiated to evaluate the safety and efficacy of HCV treatments that contain sofosbuvir. Results from four trials (FUSION, FISSION, POSITRON and NEUTRINO) are summarized below.

**8.4.4.1. FUSION:** In June 2012, the randomized, double-blind trial was initiated in 200 treatment-experienced individuals with GT2 and GT3 who were assigned to two treatment groups to receive sofosbuvir (400 mg daily) with **RBV** (1,000 mg or 1,200 mg) for duration of 12 or 16 weeks. The study also included persons with compensated cirrhosis at baseline. In the 12-week treatment group the SVR12 rate was 86% for GT2 and 30% for GT3 infected subjects. In the 16-week treatment group SVR12 rate was 94% for GT2 and 62% for GT3 subjects. No adverse side effects were observed that were attributed to sofosbuvir treatment (Gilead, 2013d).

**8.4.4.2. FISSION:** This randomized, active-controlled study, initiated in December 2012, aimed to compare 12-week course with sofosbuvir 400 mg/**RBV** with 24-week standard of care treatment **IFN/RBV** treatment in genotype 2 and 3 treatment-naïve persons. The study met its primary efficacy end point with 67% of subjects achieving SVR12 in both groups. All common adverse effects (≥ 10%) occurred more frequently in **IFN/RBV** arm. Most frequently observed adverse events (≥ 10%) in the sofosbuvir/**RBV** group were fatigue, headache, nausea, insomnia and dizziness (Gilead, 2013a).

**8.4.4.3. POSITRON:** This randomized, double-blind, placebo controlled study was initiated in March 2012 and was designed to evaluate sofosbuvir plus **RBV** for persons who were **IFN**-intolerant or ineligible/unwilling to take **IFN**. Two hundred and seven subjects with GT2 and GT3 HCV infections were enrolled. Of those, 15% had compensated cirrhosis and 53% were GT2. Among individuals with GT2, 93% achieved SVR12, among those with

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GT3 and the small percentage that had cirrhosis, 61% also achieved SVR-12 (Gilead, 2012); however the overall SVR12 rate was 78% in GT3 and GT4 combined.

8.4.4.4. NEUTRINO: This study was initiated in June 2012 in 327 treatment-naïve subjects with GT1, GT4, GT5 and GT6 to evaluate treatment outcomes of a 12-week course of sofosbuvir with **IFN** and **RBV** treatment. Overall, triple combination was found to be superior with 90% of subjects achieving SVR12 compared to a predefined historic control SVR rate of 60 percent. The most common adverse events (reported in >20%) were fatigue, headache, nausea, insomnia and anemia (Gilead, 2013a).

### 8.5. Conclusions

Sofosbuvir, **PSI-7977** (now **GS-7977**) is the most promising pan-genotypic antiviral drugs against HCV infection identified to date. In combination with SOC or other DAAs such as protease and NS5A inhibitors (see below), it demonstrated high percentage of cure rate among all GTs. Like earlier, anti-HCV nucleoside analogs sofosbuvir showed a clear synergistic effect when dosed in combination with **RBV**. Two small and separate early clinical studies showed that **RBV** could lower HCV viral load, but did not cure any subjects tested (Di Bisceglie et al., 1992; Reichard et al., 1991). In our hands, **RBV** has no anti-HCV activity in the Huh7 replicon system suggesting that the antiviral effect may be due to a host effect (Bassit et al., 2008).

SVR rates were consistently lower among GT3 patents in phase III studies reporting preliminary data. Early *in-vitro* genotype studies with sofosbuvir found similar $EC_{90}$ values for inhibition of GT1b (Con1)-, GT1a (H77)-, and GT2a (JFH-1)-derived replicons and for GT1b chimeric replicons containing the NS5B region from the J6 GT2a isolate and from GT2b and GT3a isolates (Lam et al., 2012). Thus, there was no published preclinical indication of lower efficacy versus GT3 and it remains to be seen if this pattern holds true as more data are disclosed.

On April 8, 2013, based on these clinical studies, Gilead submitted a new drug application to U.S. FDA for the use of sofosbuvir and **RBV** as an all-oral regimen in GT2 and three individuals and in combination with **IFN/RBV** for the treatment-naïve persons with GT1, 4, 5 and 6 HCV infection (Gilead, 2013c). On October 25, 2013, an FDA advisory committee voted unanimously (15 to 0) in favor of approval of the use of sofosbuvir in persons infected with GT2 and GT3 in combination with **RBV**. The committee also recommended (15-0) sofosbuvir in combination with **IFN** and **RBV** for treatment of chronic HCV in treatment-naïve adults with GT1 and GT4 infections.

## 9. Combinations of sofosbuvir with other DAAs

### 9.1. Sofosbuvir (PSI-7977; GS-7977) plus daclatasvir (BMS-790052)

The all-oral, **IFN/RBV**-free, combination of direct acting antivirals (DAA) involving sofosbuvir an NS5B polymerase inhibitor, and daclatasvir (**BMS-790052**) (Fig. 12), an NS5A inhibitor, has been explored as an attractive approach toward HCV treatment (Sulkowski et al., 2012a; Sulkowski et al., 2012b).

**9.1.1. Phase IIa**—The first combination study of sofosbuvir and daclatasvir with or without **RBV** was reported in a phase IIa trial (AI444-040 study) in treatment-naïve subjects with GT1a/1b, GT2, or GT3 HCV infection (Sulkowski et al., 2012a). Eighty-eight subjects were enrolled to receive sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combinations with or without **RBV** for 24 weeks. HCV RNA levels were monitored up to 48 weeks after discontinuation of antiviral therapy. Based on the interim results, overall,

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more than 95% of all treatment-naïve subjects with any GT tested achieved SVR4, but in GT1a/1b subjects the SVR4 was 100% (HCV RNA < 10 IU/mL). The combination of sofosbuvir and daclatasvir was generally well tolerated, and, of special significance, **RBV** only contributed to adverse effects and had no effect on virologic response.

An additional eighty-two GT1a/1b subjects were randomized to the AI444-040 study to receive sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combination with or without **RBV** for 12 weeks. In a recent update of the study (Sulkowski et al., 2012b), with a total of 170 treatment-naïve individuals with GT1a/1b, GT2, or GT3 HCV infection enrolled to receive sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combinations with or without **RBV** for 24 weeks, in GT1a/1b, GT2, or GT3 and 12 weeks in GT1a/1b more than 93% of all treatment-naïve subjects achieved SVR. Importantly, 43 out of 44 (98%) GT1 subjects achieved SVR24 and 41 out of 44 (93%) of GT2 and GT3 subjects achieved SVR24. *IL28B* GT, viral subtype, and the administration of **RBV** had no effect on virologic response. The combination of sofosbuvir and daclatasvir were generally safe and well tolerated. The development of anemia in ≤ 21% subjects was only observed with **RBV** treatment.

**9.1.2. Phase II**—The safety and efficacy of sofosbuvir (400 mg QD) and daclatasvir (60 mg QD) in combination with or without **RBV** for 24 weeks was evaluated in fourty-one GT1 subjects who experienced virologic failure with previous telaprevir- or boceprevir-based triple therapy. Sofosbuvir plus daclatasvir arm and sofosbuvir plus daclatasvir plus **RBV** arm demonstrated high SVR12 rate of 100% and 95%, respectively (Sulkowski et al., 2013).

### 9.2. Sofosbuvir plus ledipasvir (GS-5885)

The multipart phase II ELECTRON study aimed to evaluate the safety and efficacy of sofosbuvir-containing regimens with or without **IFN** and/or **RBV** in GT1, GT2 and GT3 individuals (Gane et al., 2013b) (See section 8.3.3.2 for additional details). One arm of this study focused on an all-oral regimen in GT1 treatment-naïve and null-responders in which subjects were treated with sofosbuvir and **RBV** over 12 weeks (Gane et al., 2013a). Other arms of the study were similar in design, but also included ledipasvir (**GS-5885**, Fig. 12), a direct acting antiviral NS5A inhibitor. Among treatment-naïve subjects (N = 25) and null-responders (N = 10) who received sofosbuvir plus **RBV** without ledipasvir, SVR12 rates were 84% and 10%, respectively. However, all individuals [treatment-naïve (N = 25) and null-responders (N = 10)] receiving ledipasvir in combination with sofosbuvir and **RBV** showed 100% SVR12 regardless of treatment history (Gane et al., 2013a). Common adverse events included anemia, depression and headache.

There are currently several ongoing phase II and phase III clinical studies for evaluating the safety, tolerability and antiviral efficacy of a once daily fixed-dose combination tablet containing ledipasvir (90 mg), and sofosbuvir (400 mg) with or without **RBV** (1,000 or 1,200 mg/day divided BID) in GT1 HCV infected subjects. The latest updates from these trials are summarized in the sections below (Gilead, 2013b).

**9.2.1. ION-1**—In October 2012, Gilead launched a phase III trial for evaluating safety and efficacy of sofosbuvir and ledipasvir with and without **RBV** for 12 or 24 weeks among treatment-naïve GT1 subjects. Each arm enrolled fifty individuals (total 200 subjects). Based on the initial results of the 12-week arms, the trial will expand to include 800 subjects (Gilead, 2013b).

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**9.2.2. ION-2**—A second phase III study for fixed-dose sofosbuvir/ledipasvir began in January 2013. The study plans to enroll 400 treatment-experienced GT1 subjects who have failed previous **IFN**-containing regimens (with or without protease inhibitors). This study will evaluate treatment response in a 12-week regimen with **RBV** and a 24-week regimen with or without **RBV** (Gilead, 2013b).

**9.2.3. LONESTAR**—A new Phase II clinical trial is now underway to study sofosbuvir/ledipasvir for 12 weeks and of sofosbuvir/ledipasvir with and without **RBV** for 8 weeks. This is the first trial to enroll hundred GT1 treatment-naïve persons for the shorter 8-week treatment duration. Treatment with sofosbuvir plus ledipasvir (with or without **RBV**) for 12 weeks will also be evaluated for treatment-experienced GT1 persons who have failed the protease inhibitor-containing regimen (Gilead, 2013b).

**9.2.4. ION-3**—In May 2013, Gilead started a Phase III, multicenter, randomized, open-label study to evaluate the safety, tolerability and antiviral efficacy of sofosbuvir/ledipasvir (400/90 mg) with or without **RBV** (1,000 or 1,200 mg/day divided BID) administered once daily for eight weeks versus sofosbuvir/ledipasvir (SOF/LDV) administered once daily for 12 weeks in treatment-naïve non-cirrhotic subjects with chronic GT1 HCV infection (Gilead, 2013e). The primary outcomes of this study are to determine the sustained virologic response twelve weeks after discontinuation of therapy (SVR12), as well as monitoring the number/frequency of adverse events and potential laboratory abnormalities.

### 9.3. PSI-7977 (GS-7977; sofosbuvir) plus simeprevir (TMC435)

Simeprevir (**TMC435**) (Fig. 12) is an HCV protease inhibitor currently in phase III clinical trials for HCV infection. In November 2011, a phase II clinical trial (the COSMOS study) evaluating the combination of sofosbuvir and simeprevir with or without **RBV** in individuals who are HCV GT1 null-responders to prior **IFN/RBV** with METAVIR stage either F0-F2 (cohort 1) or F3-F4 (cohort 2) was initiated. The study aims to randomize ninety subjects per cohort in a 2:1:2:1 ratio to the following arms: simeprevir 150 mg once daily + sofosbuvir 400 mg once daily for 24 weeks with or without **RBV**, or simeprevir 150 mg once daily + sofosbuvir 400 mg once daily for 12 weeks with or without **RBV**. For cohort 1, a safety and efficacy interim analysis was conducted when approximately 20% of the planned randomized patients reached the end of treatment or permanently discontinued treatment early. Interim data demonstrated RVR in 13/15, 8/11, 20/23, 5/10 in simeprevir with sofosbuvir and **RBV** (24 weeks), simeprevir and sofosbuvir (24 weeks), simeprevir with sofosbuvir and **RBV** (12 weeks), and simeprevir plus sofosbuvir (12 weeks), respectively (Lawitz et al., 2013a). Similar RVR rates were observed with simeprevir plus sofosbuvir + **RBV** treated for 12 or 24 weeks. However, lower RVR rates were observed without **RBV** for 12 (50%) and 24 week (72%) treatments. Interim SVR4 results from the 12 week arms of cohort 2 in the ongoing COSMOS study, including treatment-naïve or previous null-responder HCV patients all with METAVIR score F3-F4 were recently reported. Treatment for 12 weeks with simeprevir and sofosbuvir, with or without ribavirin, led to SVR4 rates of 96% and 100%, respectively (Medivir, 2013). All arms of this study at 12 weeks were generally considered safe and well tolerated. However, anemia and increased bilirubin was observed in 13.0% and 9.3% of the patients in the **RBV** containing arms, respectively. These events were not observed in the non-**RBV** containing arms.

### 9.4. Sofosbuvir (GS-7977; PSI-7977) a pyrimidine plus PSI-938 a purine nucleotide analog

For information on the phase II NUCLEAR and QUANTUM trials refer to sections 11.3.2 and 11.3.3 of this manuscript.

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## 10. R7432

In 2010, Roche initiated a phase I clinical trial of **R7432** in a dose-escalation study with sixty persons enrolled (Roche, 2010). This study was discontinued by February of 2012 (Roche, 2011a, b). Unfortunately, the chemical structure of **R7432**, the clinical trial results and an explanation for the discontinuation has not been disclosed.

## 11. PSI-938 (PSI-352938)

Purine nucleoside derivatives of mericitabine were designed to be second-generation agents with improved potency, pharmacokinetics, and resistance profiles. The guanosine derivative **PSI-938** (also known as **PSI-352938**) was a result of these studies (Chun et al., 2010; Clark, 2005; Du et al., 2009; Reddy et al., 2010; Sofia et al., 2010b). This derivative is a cyclic phosphate prodrug of a 2′-α-F-2′-β-*C*-methyl class of nucleosides. The choice for this particular prodrug was based on observations from previous studies which showed that nucleotide cyclic phosphate prodrugs were precursors of the nucleotide monophosphate with enhanced cellular permeability *in vitro* (Gunic et al., 2007).

### 11.1. Synthesis of PSI-938

Pharmasset reported a multi-kilogram process synthesis of **PSI-938** requiring no chromatography (Reddy et al., 2011; Wang et al., 2009). 2′-α-F-2′-β-*C*-methyl-2′-deoxy lactone **29** was prepared as described in Scheme 8. Benzoylation was originally used to complete the synthesis of a key intermediate similar in structure to compound **29** (Scheme 8). However, a switch to 4-chlorobenzyl protection afforded more crystalline intermediates that allowed for a multi-step synthesis that did not require any column chromatography.

The conclusion of the **PSI-938** synthesis is shown in Scheme 9. The *p*-chlorobenzoyl protected lactone **29** was reduced to lactol **30**, and it is interesting to note that upon standing the β/α anomeric mixture isomerized to the thermodynamically more stable β-lactol. An Appel reaction at low temperature then furnished bromo derivative **31**. Glycosylation of compound **31** with 2-amino-6-chloro purine followed by treatment with sodium ethoxide in ethanol provided the unprotected 6-ethoxy purine nucleoside **32**. After extensive investigation, conditions were found for the formation of the 3′,5′-cyclic phosphate **PSI-938** (*cis-R*P configuration) in high diastereoselectivity (>95%): it was finally isolated in 52% yield and greater than 99.5% diastereoselectivity after direct crystallization.

### 11.2. Preclinical development

In an HCV replicon cell assay, **PSI-938** had an $EC_{90}$ of 1.37 μM with no cytotoxicity up to 100 μM (Reddy et al., 2010). It demonstrated prolonged stability in simulated gastrointestinal fluid, simulated intestinal fluid, human plasma, and human liver S9 (*in vitro* model for liver stability). Expanded cytotoxicity data showed no cytotoxicity in a variety of cell lines and no mitochondrial toxicity. Rats treated with the compound had high levels of the triphosphate in the liver. Interestingly, **PSI-938** was equally active against the NS5B S282T mutant replicon. It also showed a high barrier of resistance and a different resistance profile than that of **RG-7128** (Lam et al., 2011). **PSI-938** was found to be metabolized by a phosphorylation pathway distinct from **RG-7128**. These promising results lead Pharmasset to nominate **PSI-938** as a preclinical candidate in June 2009 (Pharmasset, 2009).

### 11.3. Clinical development

**11.3.1. Phase I**—A phase I single ascending dose trial began in early 2010 (Pharmasset, 2010). Healthy male and female volunteers received a single dose of **PSI-938** ranging from 100 mg to 1,600 mg. No dose-limiting toxicity was identified. In August 2010, a multidose

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ascending dose trial was conducted in fourty subjects chronically infected with HCV GT1. Subjects received 100 mg QD, 200 mg QD, 300 mg QD, and 100 mg BID for seven days. **PSI-938** demonstrated potent antiviral activity with an average drop of 4 $\log_{10}$ IU/mL in mean HCV RNA from baseline. A consistent decline of HCV RNA levels was observed with no viral breakthrough. **PSI-938** was shown to be safe and well tolerated. No adverse events were reported as well as any subject discontinuations (Pharmasset, 2011c).

**11.3.2. Phase IIa, NUCLEAR**—The combination of sofosbuvir and **PSI-938** represented the first once daily dual-nucleotide **IFN/RBV**-free study for HCV treatment (Lawitz et al., 2011). Sofosbuvir, a pyrimidine, and **PSI-938**, a purine, are both NS5B polymerase inhibitors. In March 2011, Pharmasset published the results of the Phase IIa NUCLEAR trial in HCV GT1 treatment-naïve subjects. The study evaluated the safety, PK and antiviral activity of **PSI-938** and sofosbuvir administered as monotherapy or in combination for seven to fourteen days. Forty subjects were enrolled in cohorts 1–4 receiving **PSI-938** (300 mg QD) as monotherapy and/or sofosbuvir (400 mg QD) in combinations, and the treatment regimens resulted in median drops of HCV RNA that ranged from 5.0 to 5.2 $\log_{10}$ IU/mL. **PSI-938** was generally safe and well tolerated over seven to fourteen days, with no adverse events or subject discontinuations. HCV RNA rapidly declined in the first three days followed by continued decrease until end of treatment or assay limit of detection (LOD) was reached. Of the 24 subjects who received the combination treatment, 22 (92%) achieved HCV RNA levels of <15 IU/mL after fourteen days of treatment. Of note, sofosbuvir and **PSI-938** had similar HCV RNA reductions over seven days. The NUCLEAR study suggested that the combination of sofosbuvir and **PSI-938** was generally safe and well tolerated with no viral breakthrough and no significant pharmacokinetic interaction during the seven to fourteen day therapy. These promising results were followed by FDA Fast Track designation in August 2011 (Pharmasset, 2011d).

**11.3.3. Phase IIb, QUANTUM**—In September 2011, Pharmasset initiated the Phase IIb QUANTUM trial in approximately 450 adults of all viral genotypes to assess safety and SVR rates at 12 or 24 weeks for **PSI-938**, **PSI-7977** (sofosbuvir), and various combinations of the drugs with and without **RBV** (Pharmasset, 2011c). The trial evaluated interferon-free regimens of sofosbuvir 400 mg QD and **PSI-938** 300 mg QD with and without **RBV** over 12 or 24 weeks in treatment-naïve patients. The trial also evaluated **PSI-938** monotherapy. SVR24 was the primary endpoint of the trial with patients equally randomized across the following arms: 1) **PSI-938** only; 2) **PSI-938** and sofosbuvir; 3) sofosbuvir and **RBV**; 4) **PSI-938**, sofosbuvir, and **RBV** and 5) a placebo control of 24 weeks.

Unfortunately, routine safety monitoring detected "laboratory abnormalities associated with liver function" among the 235 individuals in the study who were receiving treatment with **PSI-938** alone or in combination with sofosbuvir or sofosbuvir and **RBV** (Pharmasset, 2011a). The remaining 12 and 24-week sofosbuvir and **RBV** combination arm continued unchanged to generate data in support of the NEUTRINO trial (see section 8.4.4.4).

### 11.4. Conclusions

In December 2011, **PSI-938** was dropped from the follow-up phase IIb QUANTUM trial and further clinical evaluation due to abnormalities in liver function (Pharmasset, 2011b). No future plans for **PSI-938** have been reported. It is unfortunate that a relatively high dose of this drug was used for the main studies, since it is likely that a lower dose would have also been effective with no apparent untoward effects especially when used with sofosbuvir.

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## 12. TMC649128

### 12.1. Preclinical development

**TMC649128** (Johansson et al., 2008; Jonckers et al., 2011) is a nucleoside NS5B polymerase inhibitor and has demonstrated an attractive pre-clinical profile: it displayed *in vitro* activity across multiple HCV GTs with a high genetic barrier to resistance. It was disclosed in May 2008, when Medivir entered into an R&D agreement with Ortho Biotech Products LP, an affiliate of Tibotec, to develop medications to treat HCV infection targeting NS5B virus polymerase. Under the agreement, Medivir and Tibotec jointly performed preclinical studies to identify potential compounds. As an effort to explore 4′-azido-2′-modified cytidines as potential HCV polymerase inhibitors known for their antiviral HCV activities, Medivir's scientists designed and synthesized 4′-azido-2′-deoxy-2′-methyl-cytidine **33** (Nilsson et al., 2012) (Fig. 13), which exhibited a moderate anti-HCV activity (Johansson et al., 2008). Although all the ester prodrug derivatives only exhibited similar or slightly lower activity compared with (**33**), the isobutyryl ester (**TMC649128**) demonstrated greatly improved mean maximum plasma concentration ($C_{max}$ = 4.65 μM) and hence a larger area under the curve ($AUC_{0-t}$ = 12.7 3 μM × h) and oral bioavailability (F = 65%).

### 12.2. TMC649128 synthesis

**TMC649128** was synthesized as shown in Scheme 10: Glycosylation of **4** with silylated uracil in the presence of tin chloride allowed for the formation of compound **33**. Treatment of compound **33** with ammonia in methanol led to the clean deprotection of the benzoyl group, leading to compound **34** in quantitative yield. Protection of the 3′- and 5′-hydroxyl groups with a TIPDS protective group, followed by deoxygenation via free-radical chemistry gave compound **36** in 97% yield with selectivity of 93:7 (Li and Piccirilli, 2003). Deprotection of the TIPDS group with TBAF generated compound **37** in high yield. Nucleoside **37** was then converted to the olefin compound **38** (Verheyden and Moffatt, 1975). Treating exocyclic olefin with iodine and benzyltriethylammonium azide, followed by benzoylation, provided 4′-azido nucleoside **40** in 81% yield over two steps. Oxidative nucleophilic substitution of compound **40** with *m*-chloroperbenzoic acid/*m*-chlorobenzoic acid in DCM afforded protected azido derivative **41**, which was readily converted to 4′-azido-2′-deoxy-2′-methyl cytidine (**42**) by treatment with *1*-H-tetrazole and 4-chlorophenyldichlorophosphate followed by ammonia deprotection. Final acylation of both 3′- and 5′-hydroxy groups led to the formation of **TMC649128**.

### 12.3. Clinical development

**12.3.1. Phase Ia—**In January 2011, Medivir announced the start of phase Ia trial of the hepatitis C polymerase inhibitor **TMC649128**. This first in human, double-blind, randomized, placebo-controlled trial, was successfully completed in June 2011 to assess the safety, tolerability, and pharmacokinetics of increasing single oral doses of **TMC649128** in nine healthy volunteers. After each administration of the drug and up to four weeks after the last administration, safety assessments (blood and urine tests, blood pressure, pulse, electrocardiogram, and physical examination) were performed (Medivir, 2011b; Tibotec, 2011b).

**12.3.2. Phase Ib—**In June 2011, Tibotec initiated a phase Ib, double-blind, randomized and placebo-controlled study in GT1 HCV-infected individuals to evaluate the safety, tolerability, pharmacokinetics and antiviral activity of multiple ascending doses of **TMC649128** given as monotherapy and in combination with **IFN** and **RBV**. The pharmacokinetic/pharmacodynamic relationship for antiviral activity, active metabolite and safety of **TMC649128** and its metabolite were assessed. The short-term safety and

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tolerability of the co-administration of **TMC649128** and **IFN** plus **RBV** during multiple dosing for 14 days in treatment-naïve GT1 HCV-infected participants was explored. The short-term antiviral effect of the combination of **TMC649128** with **IFN** and **RBV** was also preliminary assessed (Medivir, 2011c; Tibotec, 2011a).

### 12.3. Conclusions

In November 2011, this study was terminated, since the antiviral activity was considered insufficient to warrant further clinical development of **TMC649128** (Medivir, 2011a).

## 13. GS-6620

### 13.1. Preclinical development

**GS-6620** (Butler et al., 2011; Butler et al., 2009; Cho et al., 2011a; Cho et al., 2011b; Cho and Wolckenhauer, 2011) is a pangenotypic, liver-targeted nucleotide prodrug developed by Gilead Sciences for the treatment of HCV. **GS-6620** was shown to have a favorable inhibitory profile *in vitro* whereby the incorporation of corresponding triphosphate resulted in chain-termination. Cell culture resistance selection assays reported the selection of the S282T mutation in the NS5B viral polymerase. This mutation conferred >200-fold resistance in *in vitro* experiments with purified recombination S282T mutant enzyme (Fenaux et al., 2011). Oral administration of **GS-6620** to animals resulted in efficient delivery of the nucleoside triphosphate metabolite into the liver (Lawitz et al., 2012b).

### 13.2. GS-6620 synthesis

The synthesis of **GS-6620** is outlined in Scheme 11 (Cho et al., 2013). The tribenzylated 2′-*C*-Me sugar **43** was efficiently connected to the heterocyclic base **44** under lithium-halogen exchange conditions. The 1′-cyano group was introduced under Lewis acid conditions to give **46** which was followed by a boron trichloride promoted debenzylation to give *C*-nucleoside analog **47**. Formation of the 5′-phosphoramidate and 3′-isobutyl ester are preformed utilizing standard nucleoside protocols to give **GS-6620**.

### 13.3. Clinical development

The favorable preclinical characteristics of this compound made it a candidate for phase I clinical trials wherein safety, tolerability, pharmacokinetics and antiviral activity were evaluated in a multi-center, randomized, placebo controlled, multiple-dose escalation first-in-man study in 2010. Out of the ninety treatment-naïve subjects enrolled in the study, twelve showed signs of mild adverse events related to treatment. Two subjects who received the highest dose of treatment (900 mg BID) had virus levels below the limit of detection at the end of the five-day dosing period.

### 13.4. Conclusions

Although **GS-6620** was safe and well tolerated over five day of administration period, it was found that exposure to the drug was markedly lower in humans as compared to pre-clinical animal studies with substantial intra- and inter-subject variability seen in all cohorts. Gilead Sciences indicated that without further optimization of drug delivery to improve PK and antiviral activity, future clinical development would not occur for **GS-6620** (Lawitz et al., 2012b).

## 14. ALS-2200 (VX-135) and ALS-2158

In June 2011, Vertex licensed both a purine and pyrimidine nucleoside prodrug inhibitors of HCV NS5B polymerase from Alios Biopharma (Vertex, 2011). Both uridine nucleoside

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analog **ALS-2200 (VX-135)** and adenosine nucleotide analog **ALS-2158** (Beigelman et al., 2010; Beigelman et al., 2012a; Beigelman et al., 2012b; Smith et al., 2012) entered phase I clinical trials in December 2011. The exact structure of these nucleoside analogs has not yet been disclosed.

### 14.1. ALS-2258 clinical development

In an early clinical study, the purine **ALS-2158** did not show sufficient antiviral activity and the study was discontinued in September 2012 (Vertex, 2012b).

### 14.2. ALS-2200 clinical development

On the other hand, preliminary seven-day viral kinetic data with the pyrimidine prodrug **ALS-2200(VX-135)** administered at 200 mg QD (Marcellin et al., 2012) demonstrated a median drop of 4.18 $\log_{10}$ (range −3.6, −5.2) in HCV RNA in combination with **RBV** in treatment-naïve chronic HCV subjects with GT1. Five of eight subjects achieved HCV RNA levels below the limit of quantification, and two of these five achieved HCV RNA levels below the limit of detection after seven days of dosing. Similar results were obtained with monotherapy of **ALS-2200** (200 mg, once-daily, seven days). Adverse events during the study were mild to moderate with no subject discontinuations.

**14.2.1. Phase II**—As of November 2012 (Vertex, 2012a, d) future multiple phase II developments of **ALS-2200 (VX-135)** were envisaged: the first one was initiated with GlaxoSmithKline's NS5A inhibitor **GSK2336805** (Vertex, 2012d) (Fig. 14) but was terminated in June 2013 when Vertex and GSK mutually decided to cease the collaboration for a phase II study and prioritized other projects. A second study is planned for the second half of 2013 with **VX-135** in combination with Janssen's protease inhibitor simeprevir (**TMC435**, Fig. 12) in patients with GT1 HCV (Vertex, 2012a). Vertex also planned to begin a study of **VX-135** in combination with protease inhibitor Incivek® (telaprevir), the company's existing drug for persons with chronic GT1 HCV. Two additional studies include evaluating the safety and efficacy of **VX-135** plus **RBV** in treatment-naïve subjects with chronic Hepatitis C and planned study to evaluate the safety, efficacy, pharmacokinetics and pharmacodynamics of **VX-135** and daclatasvir in GT1 chronic hepatitis C subjects. All of these Phase II studies will evaluate safety, tolerability and SVR12 using 12 week all oral regimens in subjects with GT1 HCV. So far, Vertex completed dosing of 100 mg and 200 mg of **VX-135** in combination with **RBV** in Europe and both doses were well tolerated with no discontinuations. No serious adverse events have been reported and no liver or cardiac safety issues have been identified. Most recently, in April 2013, Vertex started a study of 100 and 200 mg doses of **VX-135** in combination with daclatasvir (**BMS-790052**), an NS5A inhibitor being developed by BMS (Vertex, 2012c), with results expected in early 2014. However, in July 2013, the FDA notified Vertex that a partial clinical hold had been placed on Vertex's ongoing phase II US study of **VX-135** (Vertex, 2013). This partial hold prevented further evaluation of the 200 mg dose of **VX-135** due to reversible elevated liver enzymes in patients receiving 400 mg of **VX-135** in combination with **RBV** in a European phase II study. Evaluation of the 100 mg dose of **VX-135** in combination with **RBV** in the U.S. continued. In addition, the recently initiated dosing of 100 and 200 mg of **VX-135** in combination with daclatasvir as part of a phase II study in New Zealand also continued.

## 15. BMS-986094 (INX-189/INX-08189)

### 15.1. Synthesis of BMS-986094

**BMS-986094** (Chamberlain et al., 2010; McGuigan and Perrone, 2008) is a double prodrug of 2′-*C*-methylguanosine triphosphate developed by Inhibitex and later acquired by Bristol-

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Myers Squibb (Fig. 15). The synthetic route started with trimethylsilyl (TMS) triflate mediated glycosylation of tetrabenzoyl 2′-*C*-methyl sugar **4** and 6-chloroguanine. The resulting benzoylated 6-chloro nucleoside **49** was then converted to unprotected 6-*O*-methyl analog **50** by substitution of the chlorine with sodium methoxide in methanol at room temperature. For the prodrug portion, the phosphorochloridate **53** was prepared in two steps by treatment of 1-naphthol with phosphorus oxychloride in presence of triethylamine followed by reaction with *t*-Bu$CH_2$-L-valine ester. Finally, reaction of **(53)** with **(50)** was conducted in THF in presence of *N*-methylimidazole (NMI) to generate the target compound, **BMS-986094**, in 18% yield as a mixture of diastereoisomers (Scheme 12) (McGuigan et al., 2010a; McGuigan et al., 2010b). As the anti-HCV activity and estimated half-life values were very similar between the two phosphorous diastereomers, **BMS-986094** was tested as a mixture in the latter studies.

This 1-naphthyl neopentyl alanine phosphoramidate derivative of 6-*O*-methoxy-2′-*C*-methylguanosine showed potent inhibition in an HCV replicon 1b assay ($EC_{50}$ = 0.01 μM, $EC_{90}$ = 0.04 μM) and demonstrated a 500 fold greater potency than the parent 6-*O*-methoxy nucleoside (Kolykhalov et al., 2009; McGuigan et al., 2010a; McGuigan et al., 2010b). However, in comparison with other clinical anti-HCV agents, **BMS-986094** was relatively toxic in multiple cell lines, with $CC_{50}$ values of 0.32, 2.23, 2.81, and 15.0 μM in Huh7 (human hepatoma), HepG2 (human hepatoma), BxPC3 (human pancreatic), and CEM (human T lymphocyte) cells, respectively (Furman et al., 2011). Other studies showed that **BMS-986094** caused bone marrow toxicity in the low micromolar range along with toxicity in kidney, blood, skeletal muscle, CNS and intestinal cells. Additional studies suggested that **BMS-986094** could cause unique tissue specific toxicity based on *in vitro* assay results, with the lowest $CC_{50}$ values in bone marrow cells (Hutchins et al., 2009; Standring, 2012). Furthermore, in a 14-day study, no appreciable nucleoside analog-associated mitochondrial toxicity was observed in CEM and HepG2 cells treated with **BMS-986094** up to 1 μM (Vernachio et al., 2011). It is not clear why higher concentrations were not reported.

### 15.2. Clinical development

**15.2.1. Phase Ia—**In a first-in-human, single-ascending oral dose (3 mg to 100 mg or a matching placebo) phase Ia study, **BMS-986094** was well tolerated with pharmacokinetics that supported once daily dosing (Patti et al., 2011). In a separate double-blind, placebo-controlled, dose-escalation study, 70 treatment-naïve HCV GT1 subjects dosed with **BMS-986094** at 9, 25, 50, 100, 200 mg once-daily for seven days resulted in a mean decrease in viral load of −0.62, −1.02, −1.53, −2.54 and −4.25 $\log_{10}$ IU/mL, respectively. Doses of 9, 25 and 100 mg once-daily were also investigated in combination with **RBV** over seven days, producing −0.75, −1.56 and −3.79 $\log_{10}$ IU/mL reduction in viral load, respectively. The drug was well tolerated at all doses (Rodriguez-Torres et al., 2011).

**15.2.2. Phase II—**After **BMS-986094** was granted Fast-Track Status by the FDA in March 2011, various Phase II trials were scheduled to commence in 2011 to continue investigating drug combinations for longer treatment periods. After Bristol-Myers Squibb bought Inhibitex in January 2012, **INX-189** was renamed **BMS-986094**. A 12 week Phase II clinical study in GT2/3 treatment-naïve individuals, in which **BMS-986094** was dosed at either 0, 25, 50 or 100 mg in combination with **RBV** and **IFN** resulted in RVR rates of 58%, 82%, 78% and 75%, cEVR rates of 92%, 93%, 100% and 88%, and eRVR rates of 58%, 75%, 78% and 71%, respectively. No virologic breakthroughs were observed over the course of therapy (Lawitz et al., 2012c).

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### 15.3. Conclusions

The development of **BMS-986094** was discontinued as a result of a series of significant adverse events: in August 2012, a subject receiving 200 mg once daily dosing died of heart failure and another eight subjects were diagnosed with severe kidney and heart damage (Sheridan, 2012). The study was suspended and further development of **BMS-986094** was discontinued; however, a follow-up safety evaluation in subjects who participated in prior clinical studies is ongoing (BMS, 2012). The cause(s) of these serious side effects are still under investigation at this time. It is not clear if the observed toxicity was due to the nucleoside triphosphate itself or due to potential toxic metabolites, such as neopentyl alcohol, 1-naphthanol and methanol, released during the enzymatic cleavage of the prodrug moiety or because of the extremely high levels of nucleoside triphosphates produced. Cardiac toxicity for **BMS-986094** was demonstrated to be dose-related and the postulated reason for the cardiac toxicity was mitochondrial toxicity. As mentioned above, 2′-*C*-MeG-TP has been shown to be a substrate for incorporation by human mitochondrial RNA polymerase (Arnold et al., 2012). Toxicities observed during preclinical *in vitro* studies should have resulted in closer monitoring of clinical toxicities that would have led to an earlier discontinuation of the clinical study of **BMS-986094**.

## 16. Lessons Learned

To date, nucleoside inhibitors appear to be the most promising of the three classes of direct-acting antiviral agents currently in phase III clinical studies. Recent progress in the development of nucleoside prodrugs, particularly sofosbuvir, solidifies our belief that this agent could become the best-in-class and backbone of future HCV combination chemotherapy. As such, sofosbuvir is an important milestone in the fight against HCV. The unforeseen surprises noted with nucleoside inhibitors in development are drug specific and not a class effect. The de-risking events based on carefully conducted preclinical developments and clinical trials suggest that we are on the verge of a huge game changer in the field of HCV with proof-of-concept already established for an all-oral cure for HCV. It is clear that early preclinical toxicology and pharmacology can make or break a newly drug preferably before clinical trials are begun. The tragic events related to **BMS-986094** only re-emphasizes the need for researchers to fully preclinically evaluate compounds using robust predictive toxicological tests and thorough toxicity screening in various cell-based systems and particularly mitochondrial toxicity which is paramount for the success of a new chemical entity, especially for nucleoside analogs. To assess mitochondrial toxicity, the use of the newly developed mitovir (*mito*chondrial dysfunction caused by anti*vir*al ribonucleoside) score was recently proposed (Arnold et al., 2012). The mitovir score can be readily obtained during preclinical studies and may be useful to quantify the mitochondrial toxicity potential of compounds. The possibility exists that some infected persons might be more susceptible to mitochondrial toxicity when treated with nucleoside analogs due to their inherent mitochondrial or nuclear transcription dysfunction. The bar has been raised with the FDA when it comes to toxicity associated with nucleoside analogs as is reflected by their actions with **IDX184** and **VX-135**, although exact details and guidance has not yet been forthcoming.

It is also clear that the prodrug selected may enhance oral bioavailability, but may also concurrently increase toxicity by delivering the active metabolite in high levels to undesirable compartments causing significant off-target side effects and even death. Thus, for treatment of HCV infection it is important that the drug is primarily liver targeted due to first pass effect with extensive hepatic extraction and that nucleoside triphosphate production and metabolism remain largely localized in the liver, the site of HCV replication with low systemic exposure. In the context of a viral liver infection, it is ideal for the nucleoside prodrug to be quickly metabolized and preferentially activated in the liver such

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that it does not go into systemic circulation and result in off-target exposure of the nucleoside or its triphosphate. For example, the side effects that were noted with both **IDX184** and **INX-189** would indicate that the drug was escaping first pass metabolism and reaching some degree of systemic exposure. Additionally, the intracellular half-life of the nucleoside triphosphate might be more important than the amount of NTP formed. After all, the active metabolite has to persist in the liver long enough to suppress and eliminate viral load. Furthermore, an oral prodrug that is unstable in gastric fluid/GI tract or the plasma may never reach its target to deliver the nucleoside monophosphate to the liver. It is also clearer now that the most desirable nucleoside analogs are those that are pangenotypic and will be effective in all HCV infected persons, especially the difficult-to-treat persons with advanced liver disease including those with decompensated cirrhosis. It is also becoming apparent that one pill may not fit all especially for underdeveloped countries where access and cost of the best combinations may become a severe obstacle to cures (Hagan and Schinazi, 2013).

The HCV drug development tsunami will continue and be broadly felt across many areas of healthcare in the next decade. It is anticipated that the prospects of a cure for millions of infected persons will in time become possible despite the cost of treatment since we have a moral obligation to provide life saving medicines that cure globally. However, it will take courageous leadership at various levels of society and government to succeed in eradicating HCV globally (Hagan and Schinazi, 2013).

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- Nucleoside analogs target the hepatitis C (HCV) NS5B RNA-dependent RNA polymerase.
- At least twelve nucleoside analogs have reached clinical study for HCV infection.
- Nucleoside analogs have activity against the various HCV genotypes.
- Nucleoside analogs have a high barrier to resistant virus compared to other classes of inhibitors.
- Nucleoside analogs that have reached human clinical trials are the focus of this review.

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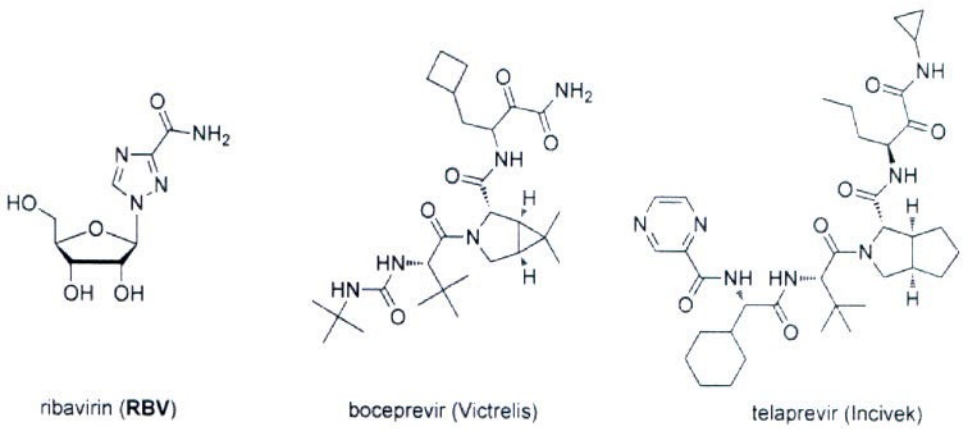



**Fig. 1.**
Structures for ribavirin (**RBV**), boceprevir (Victrelis) and telaprevir (Incivek).

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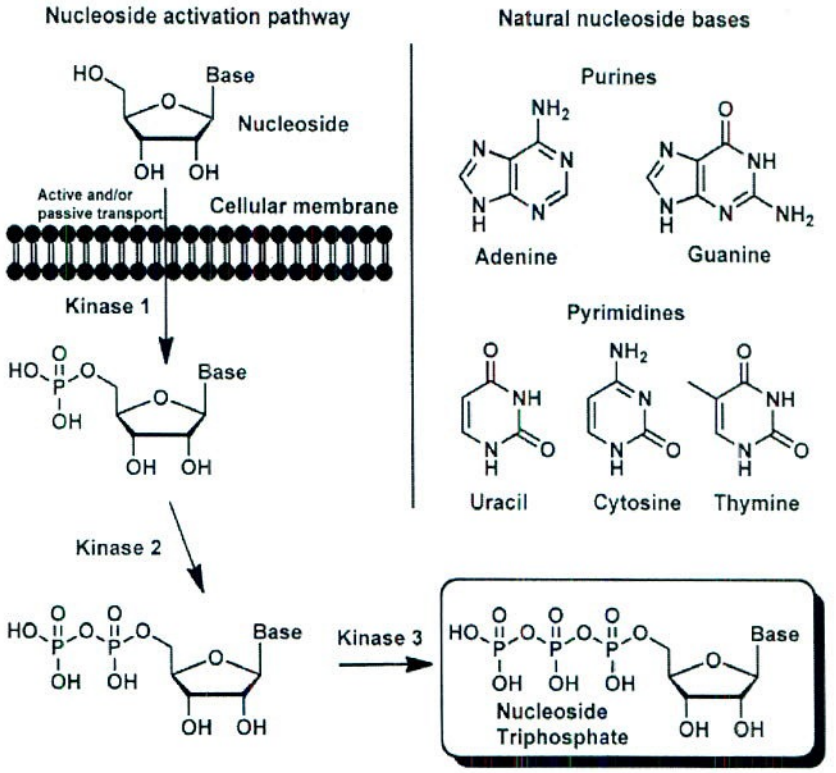



**Fig. 2.**

Nucleosides transform via three successive cellular kinases to the biologically active nucleoside analog triphosphates. Natural ribo- and 2′-deoxyribonucleoside bases.

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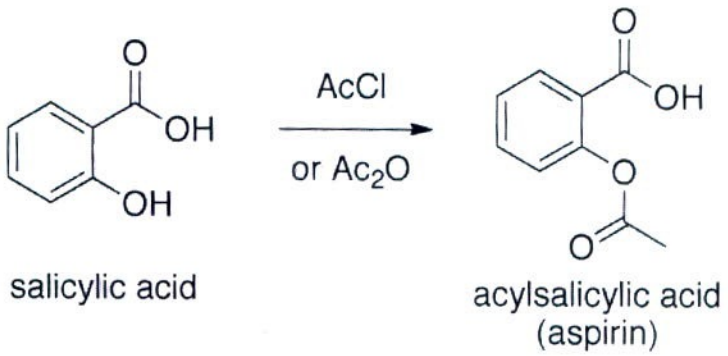



**Fig. 3.**
Synthesis of acylsalicylic acid from salicylic acid.

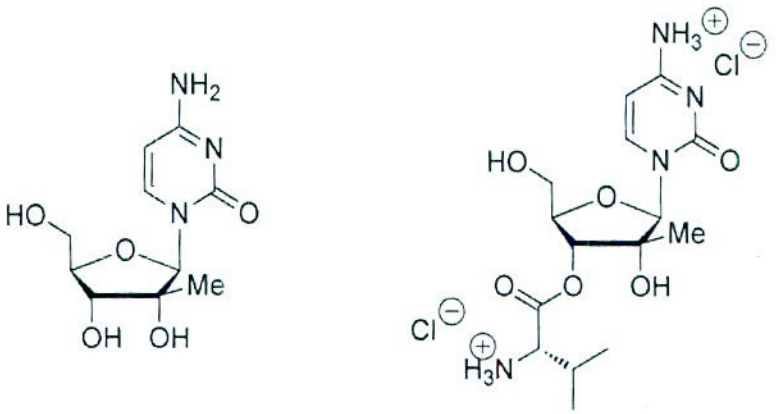



**Fig. 4.**
Chemical structures of **NM107** and **NM283**.

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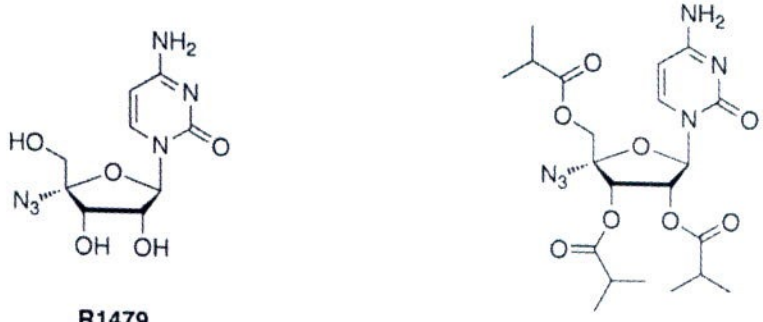



**Fig. 5.**
4′-Azidocytidine analogs used in the human clinical treatment of HCV.

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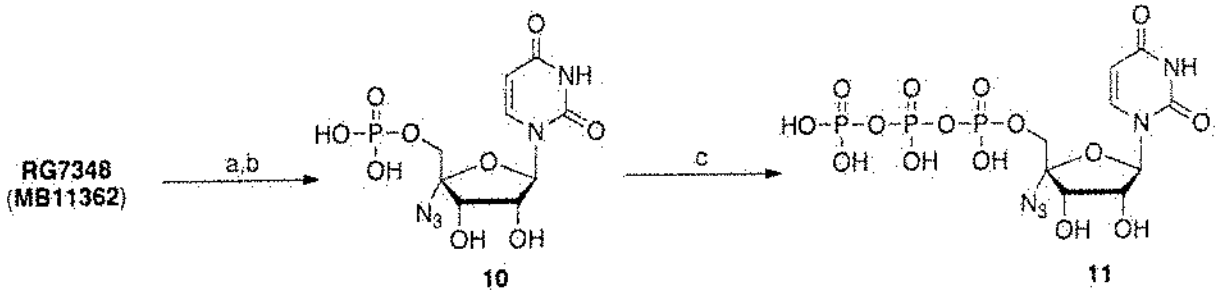



Fig. 6.
Metabolism and metabolites of **RG7348**: (a) esterases; (b) CYP3A4; (c) kinases.

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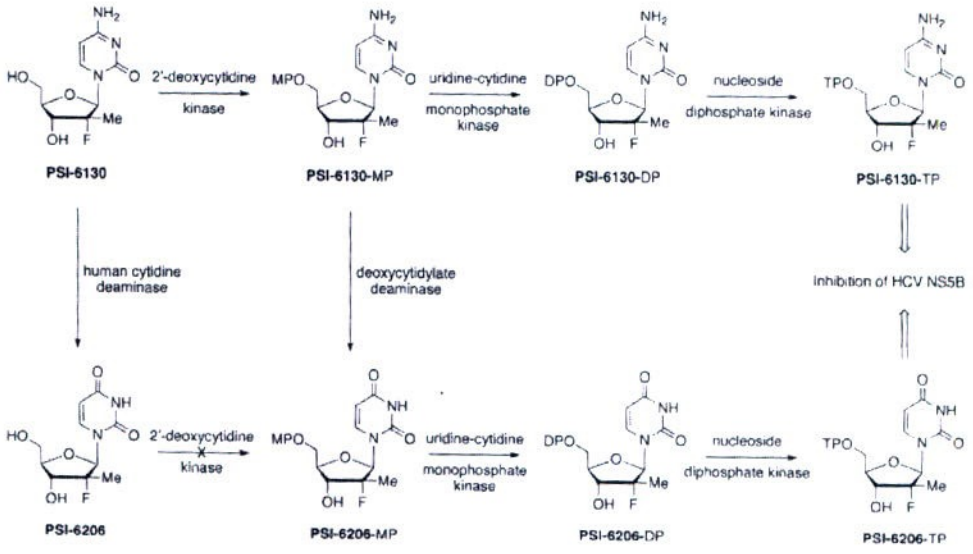



**Fig. 7.**
Metabolism of **PSI-6130** leads to both the active triphosphate and the inactive nucleoside **PSI-6206**.

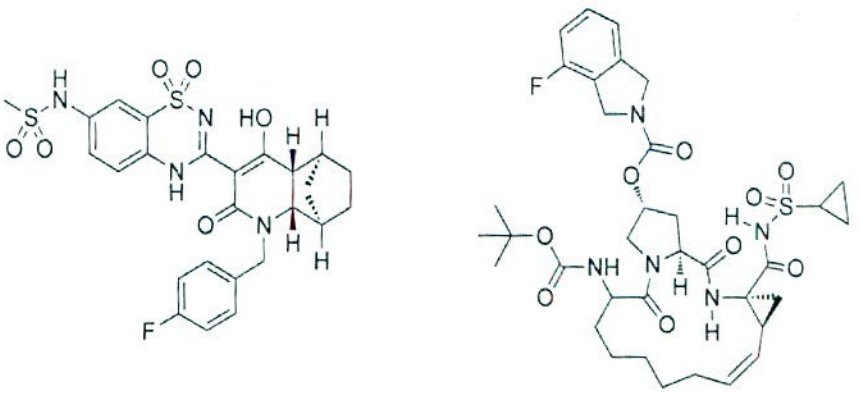



**Fig. 8.**
Structures of setrobuvir (**ANA598**, non-nucleoside polymerase inhibitor) and danoprevir (**RG7227**, protease inhibitor).

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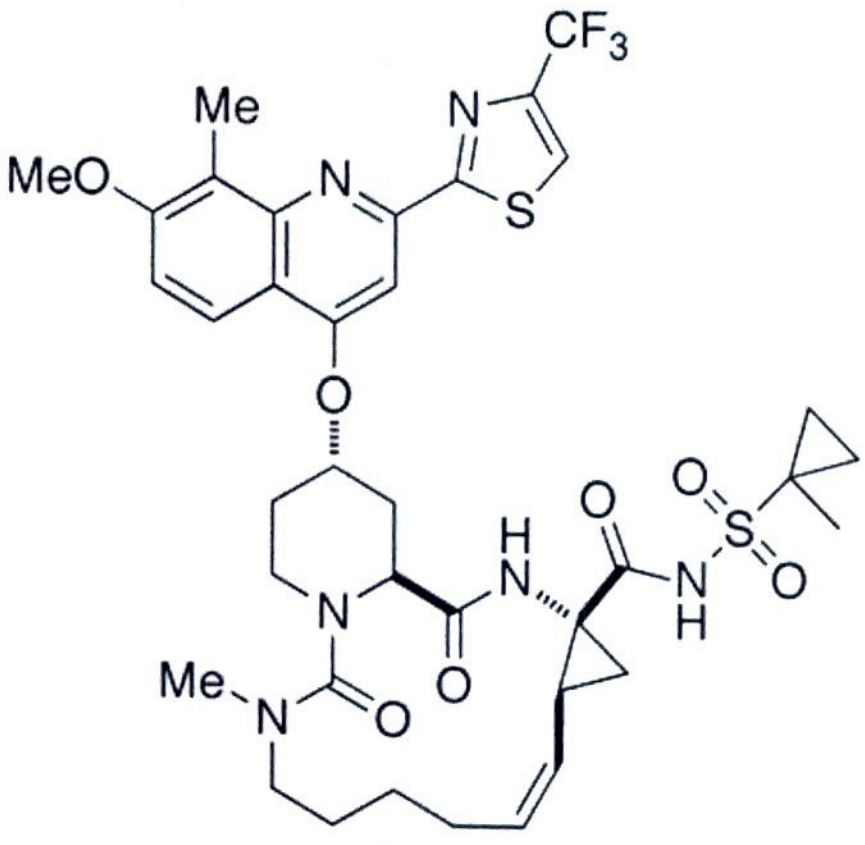



**Fig. 9.**
Chemical structure of HCV protease inhibitor **IDX320**.

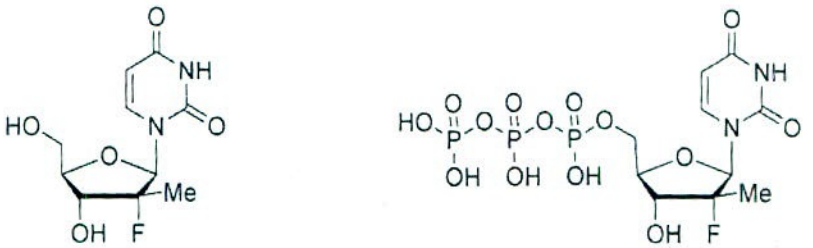



**Fig. 10.**
Structure of 2′-F-2′-*C*-methyluridine, **PSI-6206**, and its triphosphate **PSI-6206**-TP.

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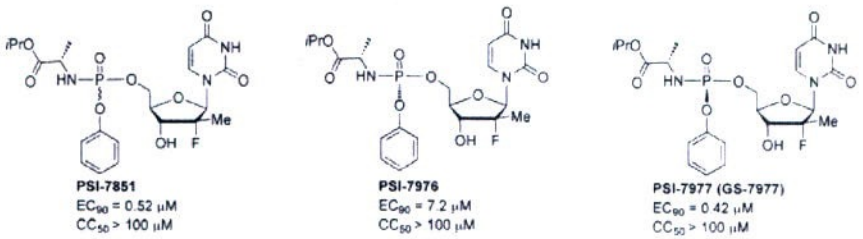



**Fig. 11.**
Structures, anti-HCV replication activity and cytotoxicity of phosphoramidate prodrug **PSI-7851** and its diastereoisomers **PSI-7976** and **PSI-7977**.



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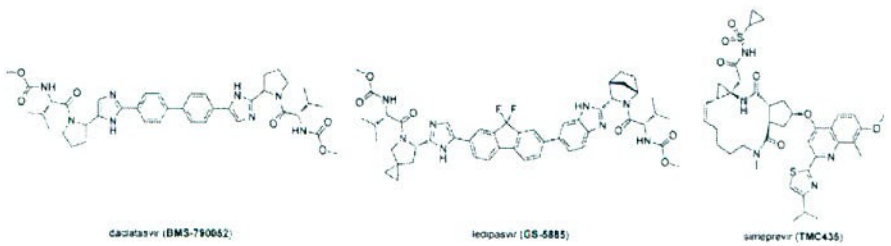
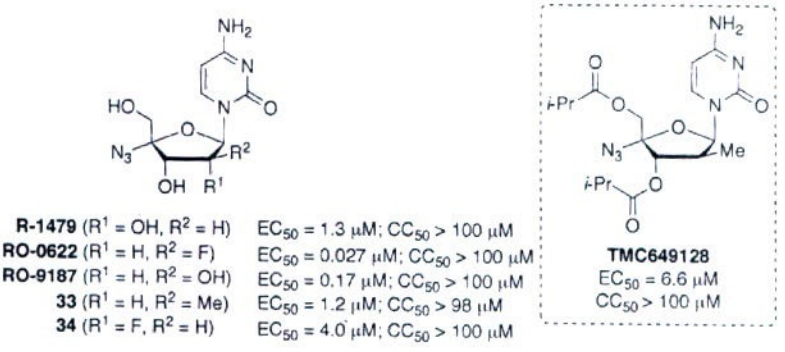



**Fig. 12.**
Structures of daclatasvir, ledipasvir and simeprevir.

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**R-1479** ($R^1$ = OH, $R^2$ = H) $EC_{50}$ = 1.3 μM; $CC_{50}$ > 100 μM
**RO-0622** ($R^1$ = H, $R^2$ = F) $EC_{50}$ = 0.027 μM; $CC_{50}$ > 100 μM
**RO-9187** ($R^1$ = H, $R^2$ = OH) $EC_{50}$ = 0.17 μM; $CC_{50}$ > 100 μM
**33** ($R^1$ = H, $R^2$ = Me) $EC_{50}$ = 1.2 μM; $CC_{50}$ > 98 μM
**34** ($R^1$ = F, $R^2$ = H) $EC_{50}$ = 4.0 μM; $CC_{50}$ > 100 μM

**Fig. 13.**
Structure of prodrug **TMC649128** and related nucleosides.



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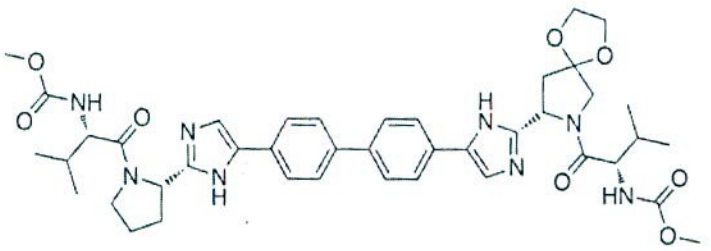


**Fig. 14.**
Structure of **GSK2336805**.

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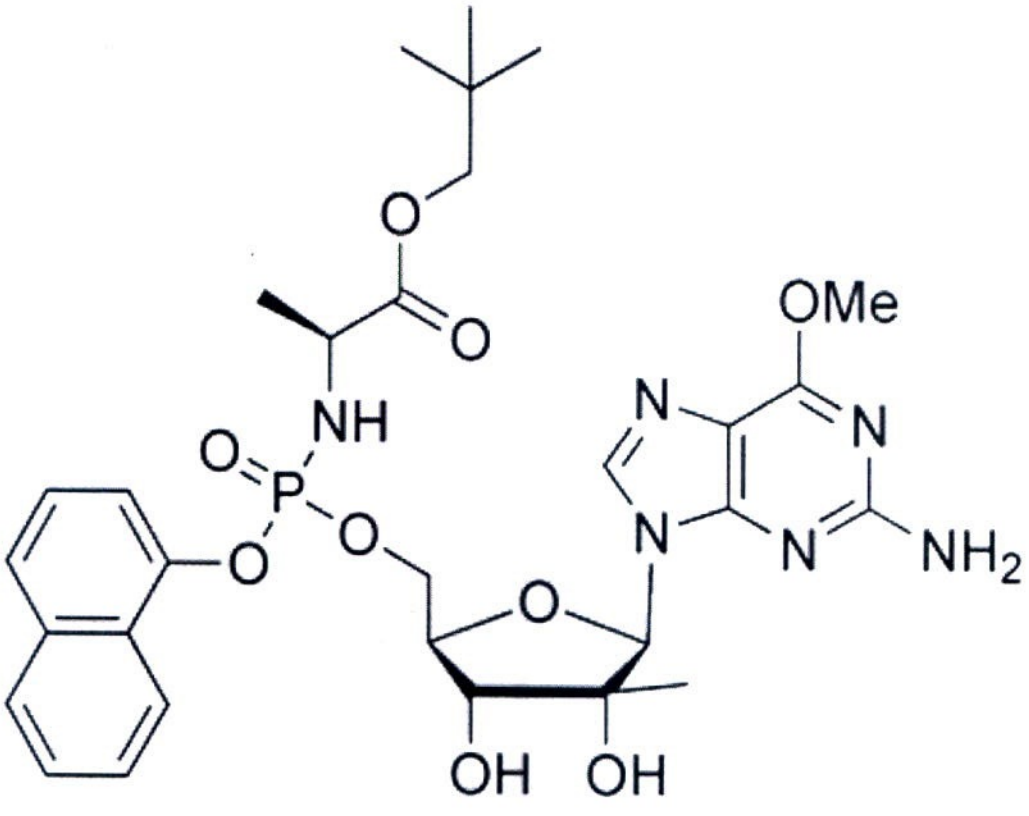
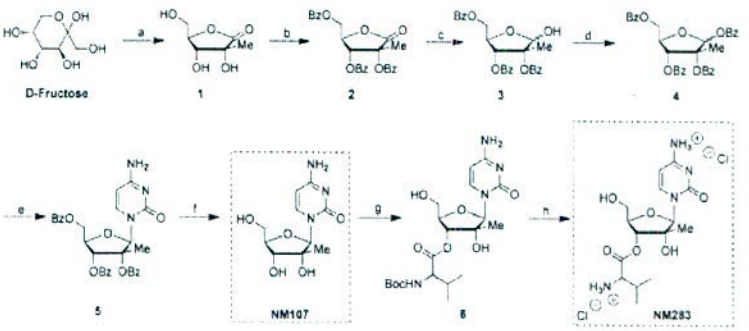



**Fig. 15.**
Structure of **BMS-986094**.

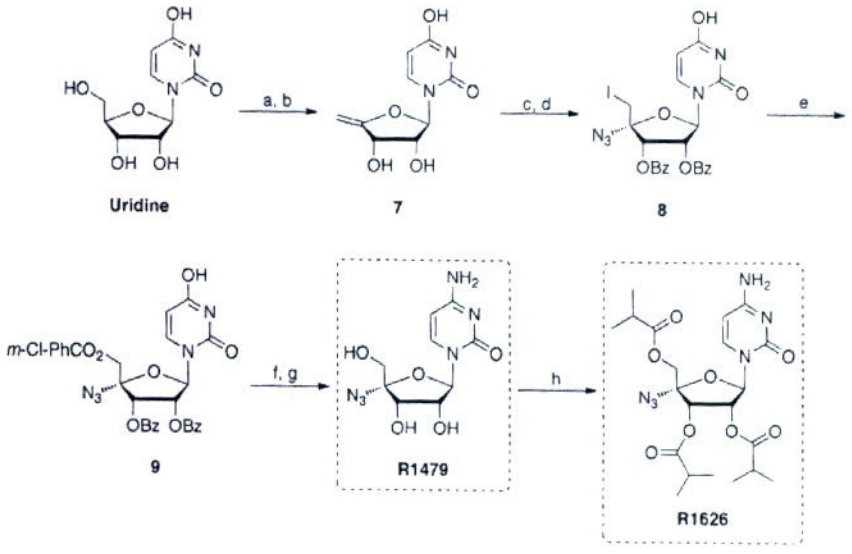



**Scheme 1.**

Synthesis of valopicitabine (**NM283**). Reagents and conditions: (a) i. CaO, water; ii. $CO_2$, oxalic acid, 18%; (b) BzCl, TEA, DMAP, DME, 83%; (c) Red-Al/EtOH, toluene, 99%; (d) BzCl, TEA, DMAP, DME, 52%; (e) bis(trimethylsilyl)acetamide, cytosine, $SnCl_4$, CAN; (f) MeONa, MeOH, r.t., 90% over two steps; (g) *N*-Boc-L-valine, CDI, DMAP, TEA, DMF/THF, 91%; (h) HCl, EtOH, 97%.



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**Scheme 2.**

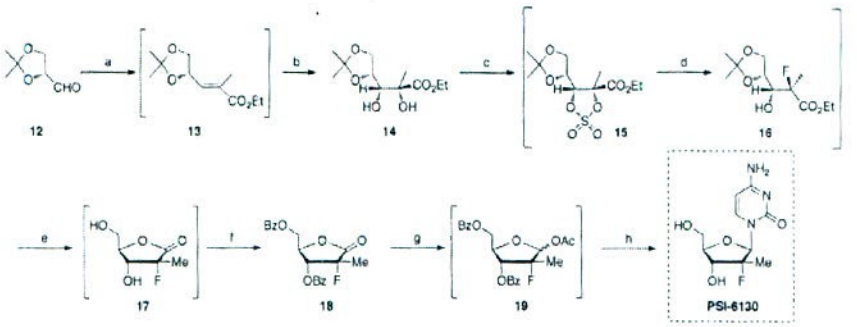
Synthesis of **R1479** and its prodrug balapiravir (**R1626**). Reagents and conditions: (a) $I_2$, $PPh_3$, imidazole, THF, 83%; (b) NaOMe, MeOH, 75%; (c) $BnN(Et)_3Cl$, $NaN_3$, 4-NMM, $I_2$, $CH_3CN$; (d) BzCl, Pyr., 74% over two-steps; (e) $K_2HPO_4$, *m*-CPBA, $CH_2Cl_2$, 71%; (f) 1,2,4-triazole, $POCl_3$, $Et_3N$, MeCN, 88%; (g) $NH_4OH$, MeOH, $H_2SO_4$-isopropanol, 95%; (h) isobutyric anhydride, KOH, DMAP, THF, 80%.



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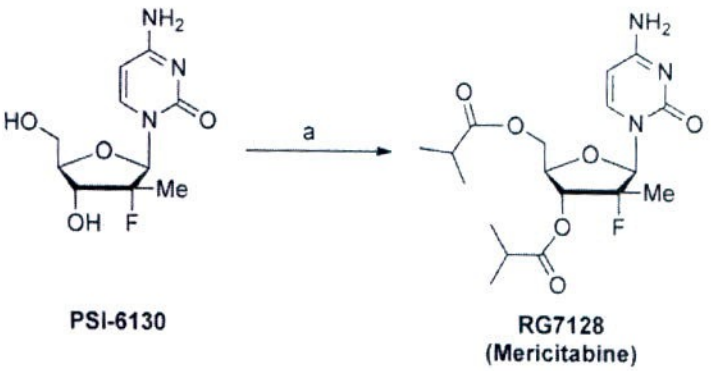


**Scheme 3.**

Synthesis of **PSI-6130**. Reagents and conditions: (a) $Ph_3PC(Me)CO_2Et$, DCM, −40°C; (b) $KMnO_4$, acetone, 0 °C, 53% over two steps; (c) i. $SOCl_2$, TEA, DCM, 0 °C; ii. aq. NaOCl, TEMPO, $NaHCO_3$, $CH_3CN$, 0 °C; (d) i. TEAF, dioxane, 100 °C; ii. $(MeO)_2C(Me)_2$, conc. HCl, dioxane, r.t.; (e) EtOH, conc. HCl, r.t.; (f) BzCl, pyridine, r.t., 47% from **(14)**; (g) i. $Li(O\text{-}tBu)_3AlH$, THF, −20 °C; ii. $Ac_2O$, DMAP, −20 °C; (h) i. silylated $N^4$-benzoylcytosine, $SnCl_4$, PhCl, 65 °C; ii. $NH_3$, MeOH, r.t.. 29% from **(18)**.



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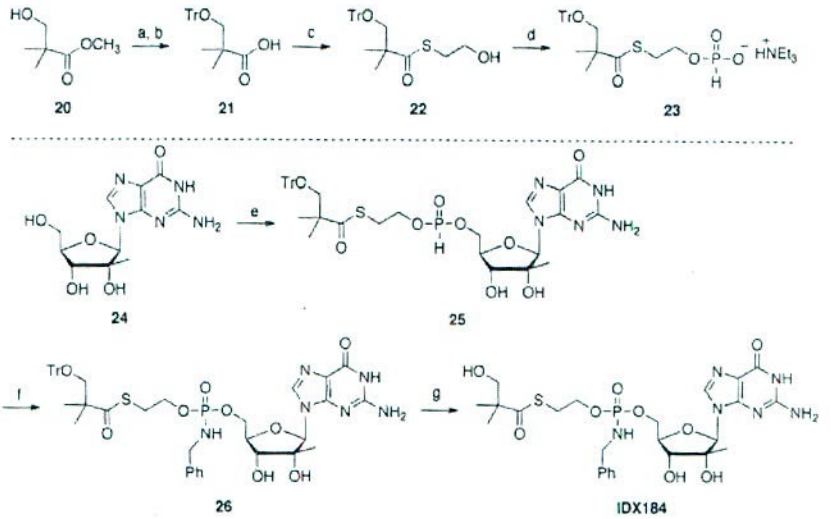



**Scheme 4.**

Synthesis of **RG7128**. Reagents and conditions: (a) DMAP, THF/$H_2O$, TEA, isobutyryl chloride, pH 6.4, 0 °C.



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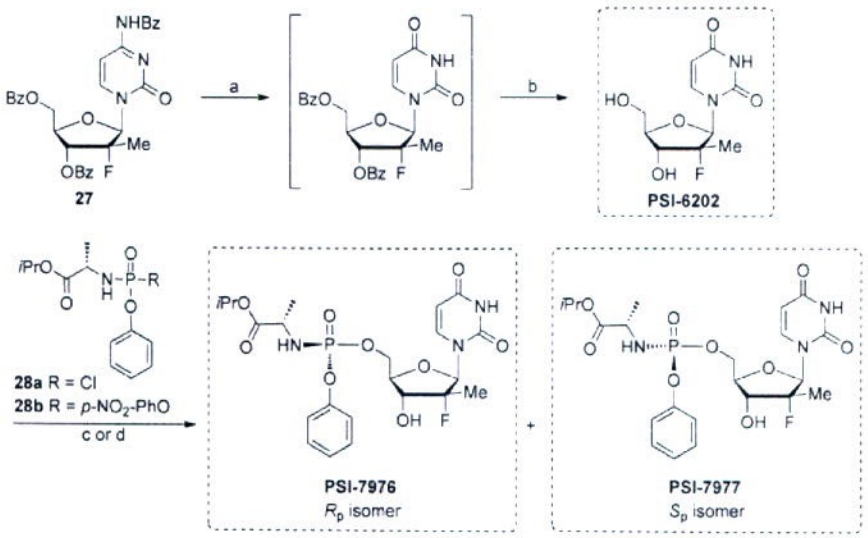


**Scheme 5.**

Synthesis of **IDX184**. Reagents and conditions: (a) TrCl, $Et_3N$, DMAP, $CH_2Cl_2$, reflux, overnight; (b) NaOH, dioxane, reflux, 16 h, 92% over two steps; (c) i. CDI, toluene/DMF (2/1, v/v), r.t., 30 min; ii. 2-mercaptoethanol, toluene/DMF (2/1, v/v), −10 °C, 3 h; (d) i. $H_3PO_3$, PivCl, pyridine, 0 °C to r.t., 3 h; ii. TEAB, 90% over two steps; (e) **(23)**, PivCl, pyridine, −15 °C, 2 h, 32%; (f) Benzylamine, $CCl_4$, r.t., 1 h, quantitative yield; (g) TFA, $CH_2Cl_2$, r.t., 30 min, 39%.



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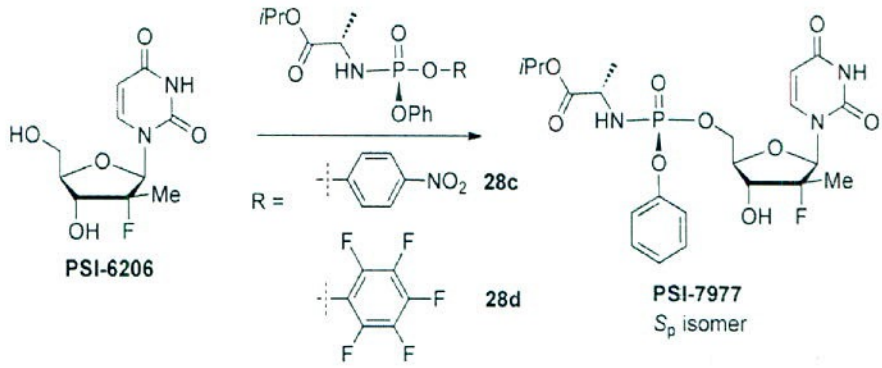



**Scheme 6.**

Synthesis of **PSI-7976** and **PSI-7977** (sofosbuvir, **GS-7977**) via column purification. Reaction conditions: (a) 70% aqueous acetic acid, 100 °C; (b) 25% methanolic ammonia, 0–15 °C, 78% overall yield; (c) **(28a)** ($R_p$:$S_p$ = 1:1) (6.5 equiv), NMI (8 equiv), $CH_2Cl_2$, r.t., overnight, 6%, 1:1 mix of **PSI-7976** and **PSI-7977**; (d) **(28b)** ($R_p$:$S_p$ ≈ 1:1) (2 equiv), *t*-BuMgCl (2.1 equiv), THF, r.t., 48 h, 47%, 3:1 mix **PSI-7977:PSI-7976**.



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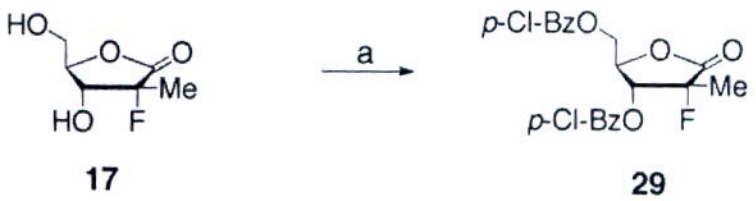



**Scheme 7.**

Synthesis of single isomer **PSI-7977** (sofosbuvir, **GS-7977**). Reaction conditions: (a) THF, *t*-BuMgCl (2.1 equiv) added over 30 min at r.t., stirred 30 min then **(28c),** r.t., 60 h, 40 %; (b) THF, *t*-BuMgCl (2.1 equiv) added over 30 min at −5 °C, stirred 30 min, warmed up to 20 °C and stirred for 30 min and then cooled to 5 °C then **(28d)** (1.2 equiv) was added over 30 min, stirred for 18 h at 5 °C, 68%.



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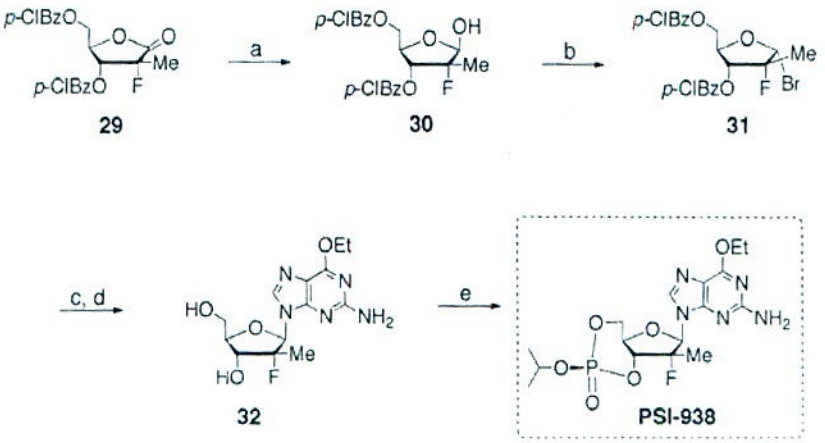



17 29

**Scheme 8.**

Synthesis of **PSI-938** intermediate lactone **29**. Reagents and conditions: (a) *p*-Cl-BzCl, pyr., r.t., 70%.

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**Scheme 9.**

Synthesis of **PSI-938**. Reagents and conditions: (a) $LiAl(t\text{-}BuO)_3H$, THF, −20 °C, 61%; (b) $CBr_4$, $PPh_3$, $CH_2Cl_2$, −20 °C, 1 h, 79%; (c) 2-amino-6-chloropurine, *t*-BuOK, *t*-BuOH, $CH_3CN$, 50 °C, 16 h, 65%; (d) NaOEt, EtOH, reflux, 1 h, 92%; (e) isopropyldichlorophosphate, NMI, $Et_3N$, $CH_2Cl_2$, 52%.

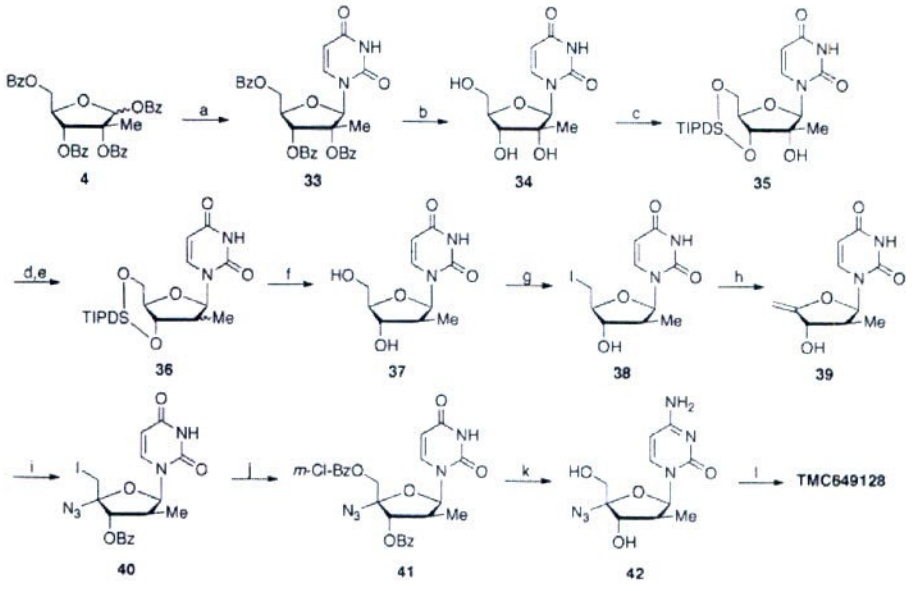


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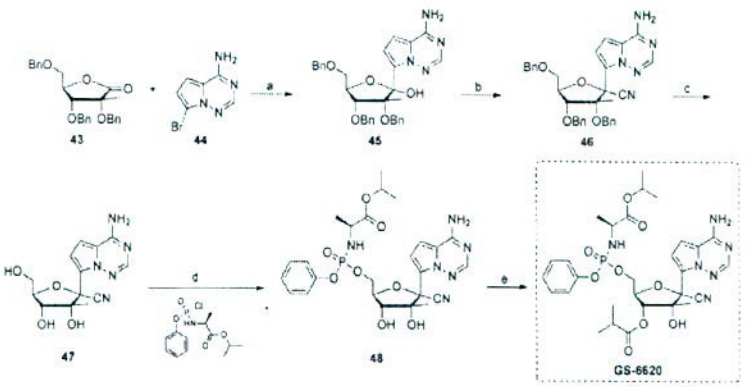
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**Scheme 10.**

Synthesis of 4′-azido-2′-deoxy-2′-methyl cytidine (**TMC649128**). Reagents and conditions: (a) bis(trimethylsilyl)uracil, $SnCl_4$, $CH_3CN$, r.t., 20 h, 96%; (b) $NH_3$, $CH_3OH$, 0–4 °C, 2 days, >99%; (c) $TIPDSCl_2$, imidazole, DMF, r.t., 2 h, 65%; (d) $ClCOCO_2Me$, DMAP, $CH_3CN$, r.t., 1 h; (e) *n*-$Bu_3SnH$, AIBN, toluene, reflux, 2 h, 97% (β/α 93:7, de 86%) over two steps; (f) TBAF/THF, r.t., 1 h, >99% (β/α 93:7); (g) $I_2$, $PPh_3$, imidazole, THF, 76%; (h) NaOMe, MeOH, 75%; (i) i. $I_2$, NMO, $Bn(Et)_3NN_3$; ii. BzCl, NMP, DMAP, THF, 81% over 2 steps; (j) *m*-CPBA, $(NH_4)_2SO_4$, DCM, 68%; (k) i. pyridine, 2-chlorophenyldichlorophosphate, 1-*H*-tetrazole; ii. dioxane, $NH_3$; iii. $NH_3$, MeOH, 61% over 3 steps; (l) *i*-PrCOCl, TEA, DCM, r.t., >99%.



**Scheme 11.**

Synthesis of **GS-6620**. Reagents and conditions: (a) **(44)**, 1,1,4,4-tetramethyl-1,4-dichlorodisilylethylene (1.0 equiv) and *n*-BuLi (3.3 equiv), THF, −78 °C followed by addition of **(43)**, 1 h, 65–80%; (b) TMSCN (6 equiv), TMSOTf (4 equiv), $CH_2Cl_2$, −15 to 0 °C, 2 h, 85–95%; (c) $BCl_3$ (3 equiv), $CH_2Cl_2$, 0 °C, 1 h, 90%; (d) *N*-methylimidazole, trimethylphosphate and THF, 0 °C to r.t., 2 h, overall yield 59%; (e) i. *N*,*N*-dimethylformamide dimethyl acetal, $CH_3CN$, r.t., 1 h; ii. isobutyric acid, DCC, $CH_3CN$, r.t., 48 h; iii. water-TFA, 0 °C to r.t., 64 h, overall yield 73%.

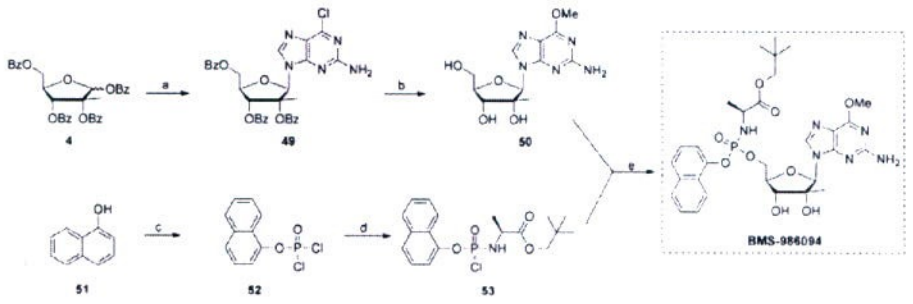


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**Scheme 12.**

Synthesis of **BMS-986094**. Reagents and conditions: (a) 2-amino-6-chloropurine, DBU, MeCN, TMSOTf, 65 °C, 4 h, 79%; (b) NaOMe, MeOH, r.t., 24 h, 76%; (c) $POCl_3$, $Et_3N$, $Et_2O$, −78 °C, 1 h then r.t., overnight, 95%; (d) $t$BuCH$_2$-L-valine ester, $Et_3N$, DCM, −78 °C, 1 h then r.t., 2 h; (e) NMI, THF, r.t., overnight, 18%.

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**Table 1**

Participants with HCV infection and results of the ELECTRON study.

| Cohort # | # of Patients | Genotype | sofosbuvir (weeks) | RBV (weeks) | IFN (weeks) | SVR (%) |
|---|---|---|---|---|---|---|
| 1 | 10[a] | GT2 and 3 | 400 mg QD (12) | 1,000 mg BID (12) | (4) | SVR24 (100) |
| 2 | 10[a] | GT2 and 3 | 400 mg (12) | 1,000 mg BID (12) | (8) | SVR24 (100) |
| 3 | 10[a] | GT2 and 3 | 400 mg (12) | 1,000 mg BID (12) | (12) | SVR24 (100) |
| 4 | 10[a] | GT2 and 3 | 400 mg (12) | 1,000 mg BID (12) | none | SVR24 (100) |
| 5 | 10[a] | GT2 and 3 | 400 mg (12) | none | none | SVR24 (60) |
| 6 | 10[a] | GT2 and 3 | 400 mg (8) | 1,000 mg BID (8) | (8) | SVR24 (100) |
| 7 | 25[a] | GT2 and 3 | 400 mg (8) | 1,000 mg BID (8) | none | SVR24 (100) |
| 8 | 10[a] | GT2 and 3 | 400 mg (8) | 800 mg BID (8) | none | SVR24 (60) |
| 9 | 25[b] | GT2 and 3 | 400 mg (8) | 1,000 mg BID (8) | none | RVR (100)<br>SVR12 (68) |
| 10 | 10[c] | GT1 | 400 mg (12) | 1,000 mg BID (12) | none | SVR12 (10) |
| 11 | 25[a] | GT1 | 400 mg (12) | 1,000 mg BID (12) | none | SVR12 (84) |

[a] Treatment-naive patients;

[b] Treatment-experimented patients;

[c] Null-responders

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Short communication

# Anti-hepatitis C virus activity of novel β-D-2′-*C*-methyl-4′-azido pyrimidine nucleoside phosphoramidate prodrugs

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**Background:** 2′-*C*-methyl and 4′-azido nucleosides have previously demonstrated inhibition of hepatitis C virus (HCV) replication by targeting the RNA-dependent RNA polymerase NS5B. In an effort to discover new and more potent anti-HCV agents, we envisioned synthesizing nucleoside analogues by combining the 2′-*C*-methyl-moiety with the 4′-azido-moiety into one molecule.

**Methods:** 2′-*C*-methyl-4′-azido pyrimidine nucleosides were synthesized by first converting 2′-*C*-methyl ribonucleosides to the corresponding 4′-exocyclic methylene nucleosides. Treatment with iodine azide, benzoylation of the 2′- and 3′-hydroxy groups, oxidative displacement of the 5′-iodo group with meta-chloroperoxybenzoic acid, and debenzoylation gave the desired 2′-*C*-methyl-4′-azido uridine and thymidine analogues in good yield. Standard conversion of uridine to cytidine via the 4-triazole yielded 2′-*C*-methyl-4′-azido cytidine. In addition, 5′-phosphoramidate derivatives of 2′-C-methyl-4′-azido uridine and cytidine were synthesized to bypass the initial phosphorylation step.

**Results:** The prepared nucleosides and their 5′-monophosphate prodrugs were evaluated for their ability to inhibit replication of the hepatitis C virus in a subgenomic replicon cell based assay. Cytotoxicity in Huh7 cells was determined simultaneously with anti-HCV activity by extraction and amplification of both HCV RNA and ribosomal RNA. Among the newly synthesized compounds, only the 5′-monophosphate nucleoside prodrugs had modest and selective anti-HCV activity. All prepared pyrimidine nucleosides and 5′-monophosphate nucleoside prodrugs displayed no evidence of cytotoxicity at high concentrations.

**Conclusions:** This work is the first example of both inactive uridine and cytidine analogues of a nucleoside being converted to active anti-HCV nucleosides via 5′-monophosphate prodrugs.

## Introduction

Twenty years ago, hepatitis C virus (HCV) was discovered by scientists at Chiron (now Novartis) and the Centers for Disease Control and Prevention to be the causative agent of non-A and non-B hepatitis in humans. Today, this virus has infected an estimated 3% of the global population and 3–4 million individuals become newly infected each year [1,2]. HCV infections often lead to reduced liver function, cirrhosis, and hepatocellular carcinoma, and eventually liver transplantation. The current approved therapy based on pegylated interferon alone or in combination with ribavirin is effective in only approximately half of the genotype 1 population [3,4]. Moreover, this limited efficacy is often associated with significant side effects leading to discontinuation of treatment [5–7]. Therefore, there is a need for the development of more effective therapeutic agents for the treatment of HCV infection.

The HCV RNA-dependent RNA polymerase (NS5B) and NS3/4A protease are currently the most promising targets for the development of novel treatments [8–11]. The activity of these virally encoded enzymes is essential for HCV replication, and antiviral agents targeting these enzymes are in both preclinical and clinical development. To date, most of the reported nucleoside analogues that inhibit HCV polymerase have modifications at the 2′ or 4′ positions of the sugar [1–12].

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Several 2′-C-methyl nucleoside analogues [13–16] and 4′-azido cytidine analogues [17,18] have been identified as potent inhibitors of HCV NS5B polymerase (Figure 1).

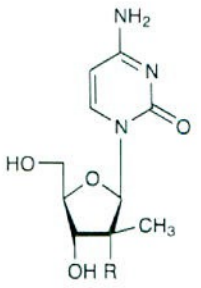
In an effort to discover new and more potent anti-HCV agents, we envisioned synthesizing a nucleoside analogue by combining the 2′-C-methyl moiety of NM-107 (1) [15] with the 4′-azido moiety of R-1479 (5) [17,18] into one molecule. Herein, we report the synthesis, anti-HCV activity and toxicity data for several 2′-C-methyl-4′-azido pyrimidines, which were synthesized as intermediates for the synthesis of the corresponding phosphoramidate derivatives. The main potential advantage of these phosphoramidate prodrugs is their ability to bypass the first phosphorylation step that is usually the rate-limiting step towards the 5′-triphosphate necessary for antiviral activity [19].

## Methods

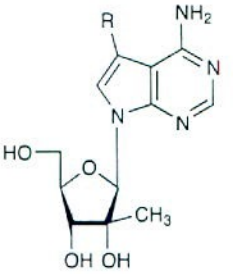
### Chemistry

Thin layer chromatography was carried out on precoated silica gel thin layer sheets 60 F254 from EMD Merck (Darmstadt, Germany). Plate layer chromatography (PLC) from Analtech, Inc. (Newark, DE, USA) was employed for purification of products. $^1$H NMR (400.14 MHz) was recorded on Varian VNMR 400 spectrometer (Varian, Inc., Palo Alto, CA, USA). Mass spectral analyses were performed on a Micromass TOF instrument (Waters Corp., Milford, MA, USA) and HPLC (Hewlett–Packard, Palo Alto, CA, USA) driven electrospray MS instrument. Analytical HPLC analyses were performed on a Hewlett–Packard HPLC with a Phenomenex Gemini-NX column (2×50 mm, 3 μm, C18, 110 Å). Mobile phase flow was 0.7 ml/min with a 3.5 min gradient from 96% aqueous media (0.05% formic acid) to 96% $CH_3CN$ (0.05% formic acid) with a 5.5 min total acquisition time and 190–360 nm photodiode array detection).

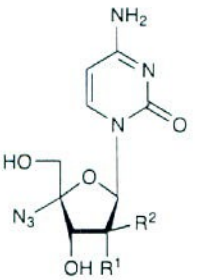
Figure 1. Structures of 2′-C-methyl and 4′-azido nucleosides



**1** R=OH (NM-107)
**2** R=F (PSI-6130)



**3** R=H (MK-0608)
**4** R=F



**5** R$^1$=OH, R$^2$=H (R-1479)
**6** R$^1$=H, R$^2$=OH (RO-9187)
**7** R$^1$=H, R$^2$=F (RO-0622)

*1-(2-Methyl-5-iodo-β-D-ribofuranosyl)uracil* (**9a**) *and 1-(2-methyl-5-iodo-β-D-ribofuranosyl)thymine* (**9b**)

2′-C-Methyluridine (5 g, 200 mmol), triphenylphosphine (8 g, 307 mmol) and imidazole (2.09 g, 307 mmol) were slurried in anhydrous tetrahydrofuran (THF). A solution of $I_2$ (5.7 g, 220 mmol) in THF was added slowly to the slurry while the reaction temperature was maintained below 28°C. The reaction mixture was stirred at room temperature for 18 h. The reaction was quenched with water and extracted with ethyl acetate. After evaporation of the solvent, the residue was purified by column chromatography using 5% methanol in dichloromethane as eluent to obtain **9a** in 85% yield. $^1$H NMR ($CD_3OD$), δ: 1.12 (s, 3H, -$CH_3$), 3.48-3.76 (m, 4H, H-3′, H-4′, H-5′), 5.73 (d, 1H, *J*=8.0 Hz, H-5), 5.93 (s, 1H, H-1′), 7.74 (d, 1H, *J*=8.0 Hz, H-6); $^{13}$C NMR δ: 4.8, 19.5, 78.0, 78.6, 80.1, 92.4, 101.5, 141.4, 150.9, 164.6.

In a similar manner from **8b** we obtained **9b** in 81% yield. $^1$H NMR ($CD_3OD$), δ: 1.12 (s, 3H, -$CH_3$), 1.88 (s, 3H, -$CH_3$), 3.53-3.71 (m, 4H, H-3′, H-4′, H-5′), 5.92 (s, 1H, H-1′), 7.62 (d, 1H, H-6); $^{13}$C NMR δ: 5.5, 11.2, 19.4, 78.0, 78.4, 79.4, 92.3, 110.3, 137.0, 151.1, 164.9.

*1-(4-Azido-2,3-O-dibenzoyl-2-methyl-5-iodo-β-D-ribofuranosyl)uracil* (**12a**) *and 1-(4-azido-2,3-O-dibenzoyl-2-methyl-5-iodo-β-D-ribofuranosyl)thymine* (**12b**)

A solution of **9a** (210 mg, 0.57 mmol) and 0.5 M sodium methoxide solution (2.84 ml, 1.42 mmol) was stirred at 60°C for 2 h. The reaction mixture was then added to a solution of *N*-methylmorpholinium mesylate in MeOH [prepared *in situ* by adding *N*-methylmorpholine (0.15 ml, 1.42 mmol) to a solution of methanesulfonic acid (0.09 ml, 1.42 mmol)]. The reaction mixture was concentrated under reduced pressure and the residue was partially purified by silica gel column chromatography (10% MeOH in $CH_2Cl_2$) to give a



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white solid product **10a** (76%). A mixture of benzyl triethylammonium chloride (194 mg, 0.855 mmol) and $NaN_3$ (55 mg, 0.855 mmol) was slurried in acetonitrile (5 ml). The insoluble NaCl was removed by filtration and the filtrate washed with acetonitrile. The acetonitrile solution of benzyltriethylammonium azide was added to a solution of **10a** and *N*-methylmorpholine (0.02 ml) in THF (5 ml), and a clear solution was formed. A solution of $I_2$ (217 mg, 0.855 mmol) in THF was added slowly at 0°C. The reaction mixture was stirred at 5–10°C for 2 h. After removal of the solvents, the residue was purified by silica gel column chromatography with 10% MeOH in $CH_2Cl_2$ to afford the product **11a** (65% yield). The benzoylation of **11a** was carried out by reaction with benzoyl chloride (0.23 ml, 1.995 mmol), triethylamine (0.277 ml, 1.995 mmol) and DMAP (14 mg, 0.114 mmol) in THF (10 ml) at room temperature overnight. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography (1–5% MeOH in $CH_2Cl_2$) to give **12a** as a foam (82%). $^1$H NMR ($CD_3OD$), δ 1.30 (s, 3H, -$CH_3$), 3.32 (s, 1H, H-3′), 3.70 (d, 1H, *J*=11.2 Hz, H-5′), 3.79 (d, 1H, *J*=11.2 Hz, H-5″), 5.74 (d, 1H, *J*=8.0 Hz, H-5), 5.98 (s, 1H, H-1′), 7.41–7.62 (m, 6H, Ar-H), 7.65 (d, 1H, *J*=8.0 Hz, H-6), 7.93–8.16 (m, 4H, Ar-H). **12b** was obtained using similar procedures in good yield, as shown in Figure 2, from **9b**. $^1$H NMR ($CD_3OD$), δ 1.82 (s, 3H, -$CH_3$), 2.02 (s, 3H, -$CH_3$), 3.34 (s, 1H, H-3′), 3.76 (d, 1H, *J*=11.2 Hz, H-5′), 3.83 (d, 1H, *J*=11.2 Hz, H-5″), 6.22 (s, 1H, H-1′), 7.38–8.16 (m, 11H, Ar-H, H-6).

*1-(4-Azido-5-O-(4-chloro)benzoyl-2,3-O-dibenzoyl-2-methyl-β-D-ribofuranosyl)uracil (***13a***) and 1-(4-azido-5-O-(4-chloro)benzoyl-2,3-O-dibenzoyl-2-methyl-β-D-ribofuranosyl)thymine (***13b***)*

To a solution of compound **12a** (170 mg, 0.275 mmol) in $CH_2Cl_2$-$H_2O$ (5:1, 24 ml), tetrabutyl ammonium sulfate (94 mg, 0.275 mmol), potassium hydrogen phosphate (94 mg, 0.412 mmol) and *m*-chloroperbenzoic acid (308 mg, 1.37 mmol) were added. The reaction mixture was stirred at room temperature overnight and then quenched with a solution of sodium thiosulfate. After stirring 5 min, a solution of sodium carbonate was added. The organic layer was separated and evaporated. The residue was purified by silica gel column chromatography using 2% MeOH in $CH_2Cl_2$ to give **13a** (62% yield). $^1$H NMR ($CD_3OD$), δ 1.31 (s, 3H, -$CH_3$), 3.32 (s, 1H, H-3′), 4.72 (s, 2H, H-5′), 5.65 (d, 1H, *J*=8.0 Hz, H-5), 5.81 (s, 1H, H-1′), 7.22–7.61 (m, 10H, Ar-H), 7.66 (d, 1H, *J*=8.0 Hz, H-6), 7.74–8.10 (m, 4H, Ar-H). **13b** was obtained by using same procedure with 66% yield. $^1$H NMR ($CD_3OD$), δ: 1.80 (s, 3H, -$CH_3$), 2.01 (s, 3H, -$CH_3$), 3.33 (s, 1H, H-3′), 3.78 (d, 1H, *J*=11.2 Hz, H-5′), 3.84 (d, 1H, *J*=11.2 Hz, H-5″), 6.23 (s, 1H, H-1′), 7.39–8.14 (m, 15H, Ar-H, H-6).

*1-(4-Azido-2-methyl-β-D-ribofuranosyl)uracil (***14a***) and 1-(4-azido-2-methyl-β-D-ribofuranosyl)thymine (***14b***)*

Compound **13a** (100 mg, 0.15 mmol) was dissolved in saturated $NH_3$ in MeOH (20 ml) and the mixture was stirred at room temperature overnight. The solvent was evaporated under reduced pressure and the residue was purified by silica gel column chromatography using 10% MeOH in $CH_2Cl_2$ to give white foam solid (40 mg, 86%). $^1$H NMR ($CD_3OD$), δ 1.19 (s, 3H, -$CH_3$), 3.67 (d, 1H, *J*=12.0 Hz, H-5′), 3.78 (d, 1H, *J*=12.0 Hz, H-5″), 4.03 (s, 1H, H-4′), 5.70 (d, 1H, *J*=8.0 Hz, H-5), 6.22 (s, 1H, H-1′), 8.04 (d, 1H, *J*=8.0 Hz, H-6); $^{13}$C NMR δ 19.9, 61.8, 73.2, 77.7, 93.2, 98.2, 101.7, 140.9, 151.0, 164.6. MS (ESI): 298 $(M-H)^-$. **14b** was obtained with same method in 83% yield. $^1$H NMR ($CD_3OD$), δ 1.15 (s, 3H, -$CH_3$), 1.84 (s, 3H, -$CH_3$), 3.64 (d, 1H, *J*=12.4 Hz, H-5′), 3.78 (d, 1H, *J*=11.6 Hz, H-5″), 4.06 (s, 1H, H-4′), 6.21 (s, 1H, H-1′), 7.93(s, 1H, H-6); $^{13}$C NMR δ 11.3, 19.8, 61.7, 73.1, 77.7, 93.1, 98.2, 110.5, 136.5, 151.2, 164.9. MS (ESI): 312 $(M-H)^-$.

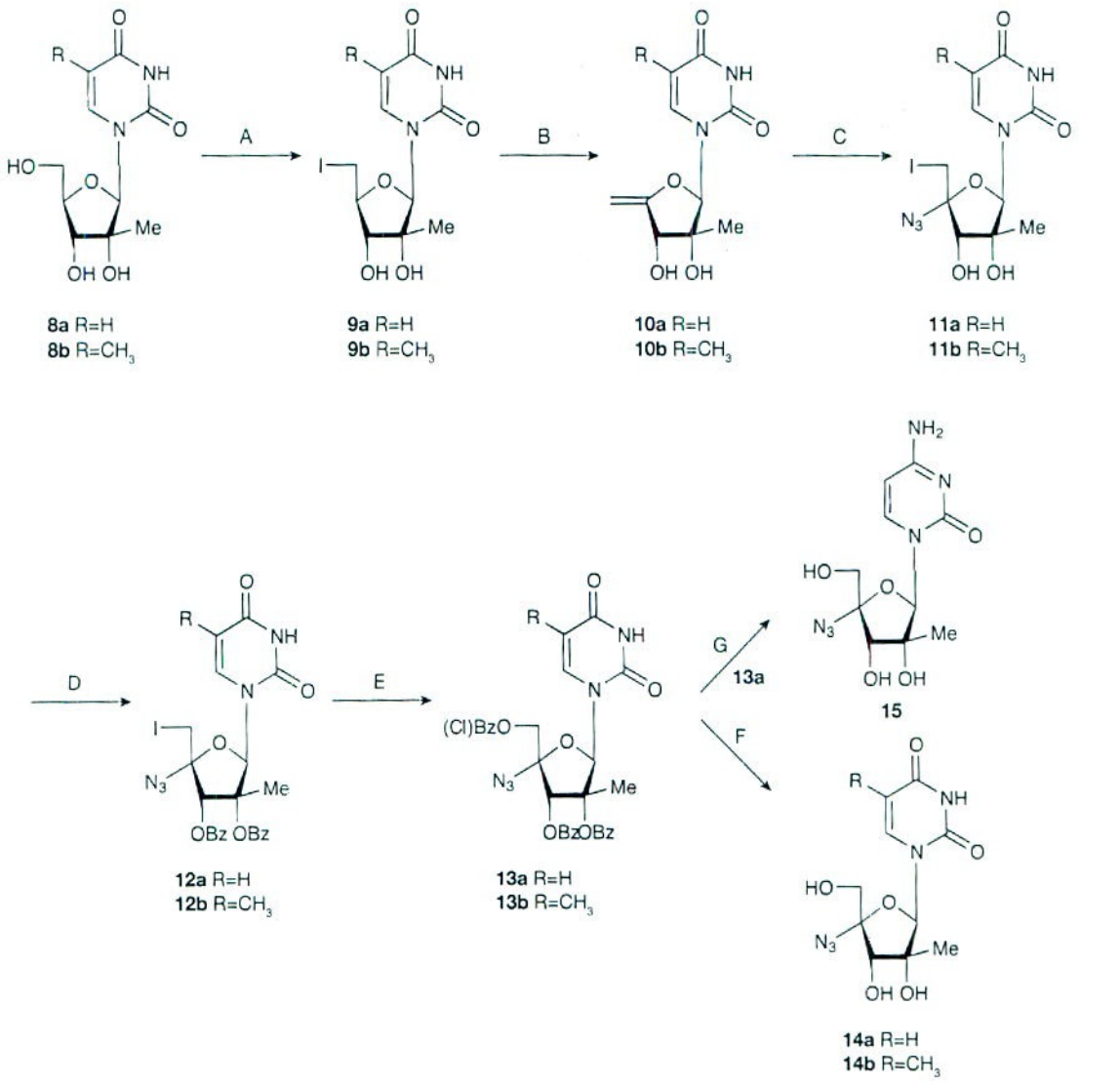
*1-(4-Azido-2-methyl-β-D-ribofuranosyl)cytosine (***15***)*

To a solution of **13a** (200 mg, 0.31 mmol), 1,2,4-triazole (339 mg, 4.91 mmol) and triethylamine (0.68 ml, 4.91 mmol) in anhydrous $CH_2Cl_2$ (30 ml) was added $POCl_3$ (0.09 ml, 0.98 mmol). The reaction mixture was stirred at room temperature for 48 h and then quenched with water. The organic layer was separated. After evaporation of the solvent under reduced pressure, the residue was dissolved in acetonitrile. A quantity of 10 ml of concentrated $NH_4OH$ was added. The reaction mixture was stirred for 2 h. The solvent was evaporated and the residue was dissolved in MeOH. A quantity of 10 ml of concentrated $NH_4OH$ was added and the reaction mixture was stirred overnight. After removed the solvent, the residue was purified by silica gel column chromatography by using MeOH/$CH_2Cl_2$ (5–30%) as eluent to give **15** in 42% yield. $^1$H NMR ($CD_3OD$), δ 1.20 (s, 3H, -$CH_3$), 3.70 (d, 1H, J=12.0 Hz, H-5′), 3.80 (d, 1H, *J*=12.0 Hz, H-5′), 4.05 (s, 1H, H-4′), 6.15 (d, 1H, *J*=7.6 Hz, H-5), 6.20 (s, 1H, H-1′), 8.50 (d, 1H, *J*=7.6 Hz, H-6); $^{13}$C NMR δ 19.7, 61.6, 72.8, 78.0, 93.7, 94.0, 98.6, 144.6, 147.4, 159.8. MS (ESI): 299 $(M+H)^+$.

*4-Azido-2-C-methyluridine-5′-O-[Phenyl(ethyloxy-l-alaninyl)]phosphate (***16***) and 4-azido-2-C-methylcytidine-5′-O-[phenyl(ethyloxy-l-alaninyl)] phosphate (***17***)*

To a solution of **14a** (70 mg, 0.23 mmol) in THF at 0°C, *t*-BuMgCl (0.47 ml, 0.47 mmol) was added and the

Figure 2. Synthetic route to 2′C-methyl-4′azido pyrimidine nucleosides




A. $I_2$, $Ph_3P$, imidazole, tetrahydrofuran (THF), rt, (85%; 9a), (81%; 9b). B. $NaOCH_3$, $CH_3OH$, 60°C, (76%; 10a), (78%; 10b). C. ICl, $NaN_3$, THF, (65%; 11a), (61%; 11b). D. BzCl, DMAP, $Et_3N$, $CH_3CN$, rt, (82%; 12a), (85%; 12b). E. meta-Chloroperoxybenzoic acid, $CH_2Cl_2$, rt, (62%; 13a), (66%; 13b). F. $NH_3$/MeOH, rt, (86%; 14a), (83%; 14b). G. Step 1: 1,2,4-triazole, $Et_3N$, $POCl_3$, $CH_2Cl_2$, 0°C to rt. Step 2: $NH_4OH/CH_3CN$/MeOH, rt, 42%.

mixture was stirred at room temperature for 20 min. A solution of phenyl-(ethoxy-L-alaninyl)-phosphorochloridate in THF (0.47 ml, 0.47 mmol) was added at 0°C and stirring was continued for 72 h. The reaction was quenched with methanol followed by aqueous $NH_4Cl$. The solvents were evaporated and the residue was purified by PLC to give prodrug **16** (3 mg, 2.3%) and 30 mg of recovered **14a**. $^1$H NMR ($CD_3OD$), δ 1.14–1.22 (m, 6H, $CH_3$-ethyl, $CH_3$-alanine), 1.33 (s, 3H, $CH_3$-2′), 3.92–4.29 (m, 6H, $CH_2$-ethyl, CH-alanine, H-3′, H-5′), 5.61 (d, 1H, *J*=8.0 Hz, H-5), 6.17 (s, 1H, H-1′), 7.17–7.37 (m, 5H, phenyl-H), 7.58 (d, 1H, *J*=8.0 Hz, H-6). MS (ESI): 553 $(M-H)^-$. The same procedure was employed for **15** (35 mg, 0.12 mmol) to prepared prodrug **17** (3 mg, 4.6%). $^1$H NMR ($CD_3OD$), δ 1.12–1.33 (m, 9H, $CH_3$-ethyl, $CH_3$-alanine, $CH_3$-2′), 3.89–4.48 (m, 6H, $CH_2$-ethyl, CH-alanine, H-3′, H-5′), 5.83 (d, 1H, *J*=7.6 Hz, H-5), 6.28 (s, 1H, H-1′), 7.17–7.38 (m, 5H, phenyl-H), 7.60 (d, 1H, *J*=7.6 Hz, H-6). MS (ESI): 554 $(M+H)^+$.



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Table 1. Anti-HCV replicon activity and cellular toxicity of synthesized nucleoside and nucleotide analogues[a]

| Compound | HCV $EC_{50}$, μM | HCV $EC_{90}$, μM | $CC_{50}$, μM | | | |
|---|---|---|---|---|---|---|
| | | | Huh7 | PBM | CEM | Vero |
| NM-107 | 2.8 | 9.6 | >10 | 29.4 | 24.5 | >100 |
| R-1479 | 10.6 | 31.8 | >10 | >100 | 10.5 | >100 |
| 14a | >10 | >10 | >10 | >100 | >100 | >100 |
| 14b | >10 | >10 | >10 | >100 | >100 | >100 |
| 15 | >10 | >10 | >10 | >100 | >100 | >100 |
| 16 | 3.0 ±1.1[b] | 7.6 ±1.7[b] | >100 | >100 | >100 | >100 |
| 17 | 4.9 ±2.3[b] | >10[b] | >100 | >100 | >100 | >100 |

[a]Values are means of triplicate measurements. [b]Means of two experiments ±SD. $CC_{50}$, 50% cytotoxicity concentration; $EC_{50}$, 50% effective concentration; $EC_{90}$, 90% effective concentration; HCV, hepatitis C virus; PBM, peripheral blood mononuclear cell.

## Virology

### *HCV replicon assays*

Huh 7 clone B cells containing HCV replicon RNA were seeded in a 96-well plate at 5,000 cells/well, and the compounds tested initially at 10 μM in triplicate immediately after seeding. Following 5 days incubation (37°C, 5% $CO_2$), total cellular RNA was isolated by using the VersaGene RNA purification kit (Gentra, Minneapolis, MN, USA). Replicon RNA and an internal control (TaqMan rRNA control reagents, Applied Biosystems, Foster City, CA, USA) were amplified in a single step multiplex real time reverse transcriptase (RT)-PCR assay. A dose–response curve was determined for nucleosides demonstrating antiviral activity below 10 μM. The antiviral effectiveness of the nucleosides was calculated by subtracting the threshold RT-PCR cycle of the test compound from the threshold RT-PCR cycle of the no-drug control (ΔCt HCV). A ΔCt of 3.3 equals a 1 $\log_{10}$ reduction (equal to 90% less starting material) in replicon RNA levels. The cytotoxicity of the compounds was also calculated by using the ΔCt ribosomal RNA (rRNA) values. (2′-Me-C) was used as the control. To determine 90% effective concentration, 50% effective concetration and 50% cytotoxicity concentrations, ΔCt values were first converted into fraction of starting material and were then used to calculate the percentage inhibition.

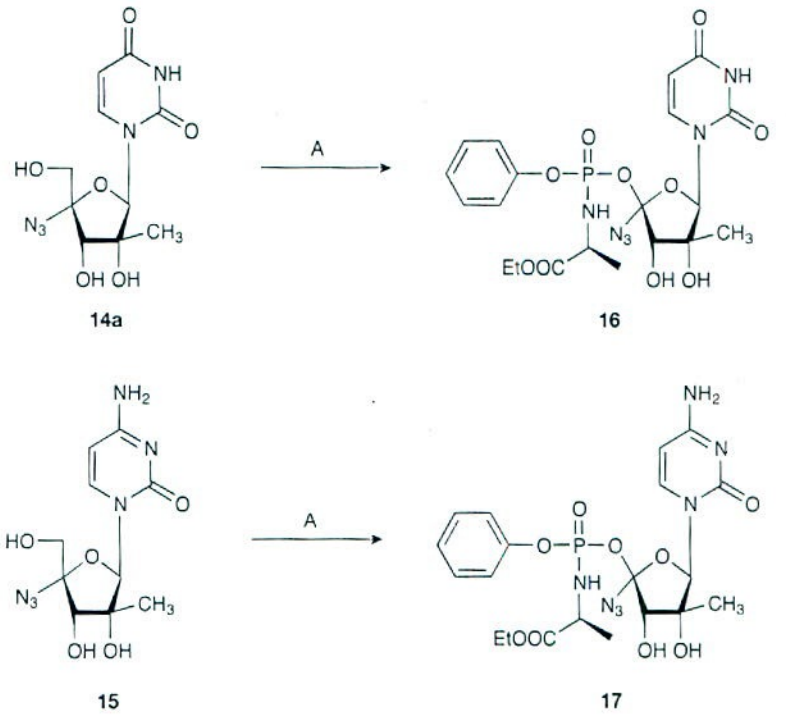
## Results

During the preparation of this paper, an alternate synthesis appeared for 2′-C-methyl-4′-azido cytidine (15) [20]. The general procedure used for the synthesis of 2′-C-methyl-4′-azido nucleosides was an extension of that previously reported in the literature (Figure 2) [17,18,21,22].

2′-C-Methyl ribonucleosides **8a–b** [23] were converted to the corresponding 5′-iodo compounds **9a–b** and then treated with sodium methoxide to yield the 4′-exocyclic methylene nucleosides **10a–b**. 4′-Azido-nucleosides **11a–b** were obtained in good yield by treatment of olefins **10a–b** with *in situ* generated iodine azide. Benzoylation of the 2′ and 3′-hydroxy groups followed by oxidative displacement of 5′-iodo group with meta-chloroperoxybenzoic acid afforded the protected 4′-azido nucleosides **13a–b**. Deprotection with $NH_3$ in MeOH gave the desired 2′-C-methyl-4′-azido uridine **14a** and thymidine analogue **14b** in good yield. 2′-C-Methyl-4′-azido cytidine (**15**) was prepared, for comparison, in two steps by treatment of compound **13a** with 1,2,4-triazole and phosphorous oxychloride in the presence of triethyl amine. Subsequent exposure of the crude 4-(*1H*-1,2,4-triazol-1-yl)-2′-C-methyl-4′-azido pyrimidinone to $NH_4OH$ furnished the desired 2′-C-methyl-4′-azido cytidine, (**15**; Figure 2). This synthesis represents an alternate and higher yielding approach to this class of compounds when compared with previous work [20].

The 2′-C-methyl-4′-azido nucleosides **14a** and **14b** were evaluated for their ability to inhibit HCV RNA replication using a Huh7-cell-based subgenomic replicon assay [24] and compared with the known cytidine analogue **15**. Cytotoxicity in Huh7 cells was determined simultaneously with anti-HCV activity by extraction and amplification of both HCV RNA and rRNA. The anti-HCV replicon activity and cytotoxicity of these compounds are summarized in Table 1. It is interesting to note that NM-107 displayed low toxicity in peripheral blood mononuclear (PBM) and CEM cells, whereas R-1479 showed some toxicity only in the more intensively proliferative CEM cell line. Although the lack of anti-HCV activity might have been predicted for the uridine analogue **14a** and the thymidine analogue **14b**, we were surprised to find that the cytidine analogue **15** was also inactive at concentrations up to 10 μM [20]. The inactivity of the cytidine analogue **15** is in clear contrast to the trend that is seen in the R-1479 [25], NM-107 [26] and PSI-6130 [27,28] compound families where the cytidine nucleoside analogues display anti-HCV activity, whereas the uridine nucleosides are devoid of anti-HCV activity. Indeed, these three new analogues displayed no

Figure 3. Reagents and conditions




A. *t*-BuMgCl, phenyl-(ethoxy-L-alaninyl)-phosphorochloridate, tetrahydrofuran, 0°C to rt, 72 h (2.3%; 16), (4.6%; 17).

inhibition of HCV replicon RNA replication or toxicity to Huh7. Further evaluation in PBM, CEM, and Vero cells up to 100 μM indicated no cytotoxicity. The lack of cytotoxicity or activity of these nucleosides as anti-HCV agents might be the result of inefficient uptake and/or their inability to be anabolized to the corresponding nucleoside triphosphates.

Because the cytidine analogue **15** is a combination of the 2′-C-methyl moiety of NM107 (**1**) [15] and the 4′-azido moiety of R1479 (**5**) [17,18] and it has been reported that the initial phosphorylation of each is the result of different cellular enzymes [18], we hypothesized that disruption in the phosphorylation pathway was a likely cause for loss of anti-HCV activity. To address this possibility, the 5′-monophosphate aryloxy phosphoramidate derivatives of uridine analogue **14a** and cytidine analogue **15** were synthesized. This phosphoramidate prodrug approach was successfully applied to various nucleosides [19,29–31] and allowed the nucleoside analogue to bypass the first phosphorylation step by intracellular delivery of monophosphorylated nucleoside. HCV phosphoramidate prodrugs have been successfully utilized to convert the inactive uridine analogue of R-1479 [25]; adenosine analogue of R-1479 [32]; uridine analogue of PSI-6130 [27,28]; and uridine analogue of NM-107 [26] into active compounds *in the* HCV replicon system.

The target monophosphate prodrugs **16** and **17** were prepared as diastereoisomeric mixtures at the phosphorous centre by the addition of phenyl-(ethoxy-L-alaninyl)-phosphorochloridate to the preformed 5′-alkoxide of nucleosides **14a** and **15** in THF (Figure 3).

In contrast to the parent nucleosides **14a** and **15** that were inactive, the prodrugs **16–17** exhibited anti-HCV activity with 50% effective concentration values of 3.0 μM and 4.9 μM, respectively. Additionally, these 5′-monophosphate prodrugs display no observable toxicity toward Huh7, PBM, CEM, or Vero cells up to 100 μM. These results suggest that the poor activity of parent nucleosides was probably the result of insufficient conversion to their corresponding 5′-monophosphate nucleoside.

## Discussion

Three 2′-C-methyl-4′-azido-pyrimidine nucleosides and two novel 5′-monophosphate prodrugs were synthesized and evaluated for their antiviral activity in an HCV replicon system. As expected, 2′-C-methyl-4′-azido cytidine **15** did not exhibit anti-HCV activity up to 10 μM [20].



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Furthermore, we prepared and tested the corresponding uridine and thymidine 2′-C-methyl-4′-azido analogues and found them also to be devoid of anti-HCV activity and all three pyrimidine analogues displayed no evidence of cytotoxicity at high concentrations. We demonstrated that the 5′-monophosphate prodrugs of the uridine analogue **14a** and cytidine analogue **15** exhibited good anti-HCV activity without apparent cytotoxicity. This work is the first example of both inactive analogues of a nucleoside being converted to active anti-HCV nucleosides via 5′-monophosphate prodrugs. Ongoing work is focused on optimizing the 5′-monophosphate prodrug portion of these novel nucleoside analogues to improve their potency.

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## Disclosure statement

RFS is the principal founder and a Director of RFS Pharma, LLC. His laboratory received no funding from RFS Pharma for this work. All other authors declare no competing interests.

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TITLE OF INVENTION: 2'-4'-DIFLUORO-2'-METHYL SUBSTITUTED NUCLEOSIDE DERIVATIVES AS INHIBITORS OF HCV RNA REPLICATION

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- ☐ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number _____ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)
- ☐ Applicant certifying micro entity status. See 37 CFR 1.29 — NOTE: Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.
- ☐ Applicant asserting small entity status. See 37 CFR 1.27 — NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.
- ☐ Applicant changing to regular undiscounted fee status. — NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature _____ Date _____

Typed or printed name _____ Registration No. _____



PTOL-85 Part B (10-13) Approved for use through 10/31/2013. OMB 0651-0033 U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

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| APPLICATION NO. | FILING DATE | FIRST NAMED INVENTOR | ATTORNEY DOCKET NO. | CONFIRMATION NO. |
|---|---|---|---|---|
| 14/365,416 | 06/13/2014 | Jing Zhang | 111890-83855 (30770 US) | 2314 |

115777 7590 01/22/2015

Jennifer L. Kisko
GrayRobinson, P.A.
301 E. Pine Street
Suite 1400
Orlando, FL 32801

| EXAMINER | |
|---|---|
| MCINTOSH III, TRAVISS C | |

| ART UNIT | PAPER NUMBER |
|---|---|
| 1673 | |

DATE MAILED: 01/22/2015

**Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)**
(Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

## OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## Privacy Act Statement

**The Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

| Notice of Allowability | Application No. 14/365,416 | Applicant(s) ZHANG ET AL. | |
|---|---|---|---|
| | Examiner TRAVISS C. MCINTOSH III | Art Unit 1673 | AIA (First Inventor to File) Status No |

***-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--***

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to *claims filed 11/17/2014*.
   ☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on _____.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on _____; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are *1-14 and 20-26*. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see http://www.uspto.gov/patents/init_events/pph/index.jsp or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

   **Certified copies:**

   a) ☐ All b) ☐ Some *c) ☐ None of the:
   1. ☐ Certified copies of the priority documents have been received.
   2. ☐ Certified copies of the priority documents have been received in Application No. _____.
   3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

   * Certified copies not received: _____.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS ( as "replacement sheets") must be submitted.
   ☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date _____.

   **Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**
6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

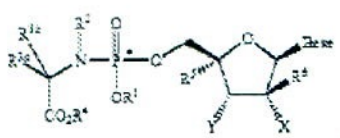
1. ☒ Notice of References Cited (PTO-892)
2. ☐ Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____
3. ☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material
4. ☐ Interview Summary (PTO-413), Paper No./Mail Date _____.
5. ☐ Examiner's Amendment/Comment
6. ☒ Examiner's Statement of Reasons for Allowance
7. ☐ Other _____.

/TRAVISS C MCINTOSH III/
Primary Examiner, Art Unit 1673

## REASONS FOR ALLOWANCE

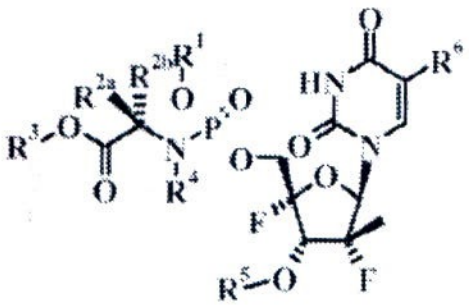
The present application is being examined under the pre-AIA first to invent provisions.

The following is an examiner's statement of reasons for allowance: the prior art is not seen to teach or fairly suggest the compounds of formula I nor methods of using the same. The closest prior art is seen to be US Patent No 7,964,580 who discloses various 2'-methyl-2'-fluoro phosphoramidite nucleosides, and teaches that F could be in the 4'-position. However, the patent does not disclose any compounds claimed herein, nor does the patent blaze a trail to the compounds claimed herein. One of skill in the art would have to pick and choose from hundreds of different variables to arrive at the combination instantly claimed. Formula I in '580 is set forth as:



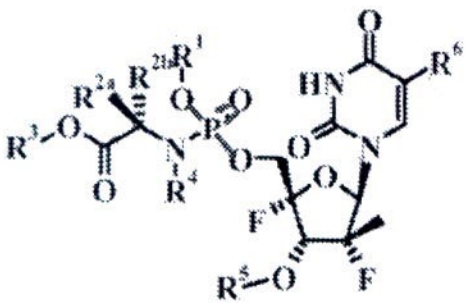
however $R^5$ is optionally H, lower alkyl, CN, vinyl, O-lower alkyl, hydroxy lower-alkyl, $CH_2F$, $N_3$, $CH_2CN$, $CH_2NH_2$, $CH_2NHCH_3$, $CH_2N(CH_3)_2$, optionally substituted alkyne, or halogen including F, Cl, Br, or I. Likewise, $R^6$, X, and Y are also moieties selected from various Markush groups. As such, the examiner does not believe a skilled artisan would be driven to select the compounds which are instantly claimed with this reference before them.

Additionally, the examiner would like to note that the compound of formula I of claim 1



which is set forth structurally as:

is being interpreted as:



wherein there is a double bond between the P-atom and O-atom, a single bond between the P-atom and the $OR^1$ group, and a single bond attaching the P-atom to the 5'-O of the sugar since this is what applicants appear to have been claiming based on the guidance of the compounds set forth in the specification.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

Any inquiry concerning this communication or earlier communications from the examiner should be directed to TRAVISS C. MCINTOSH III whose telephone number is (571)272-0657. The examiner can normally be reached on M-F 9:30-6:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Shaojia A. Jiang can be reached on 571-272-0627. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/TRAVISS C MCINTOSH III/
Primary Examiner, Art Unit 1673
January 12, 2015

| Notice of References Cited | Application/Control No. 14/365,416 | Applicant(s)/Patent Under Reexamination ZHANG ET AL. | |
|---|---|---|---|
| | Examiner TRAVISS C. MCINTOSH III | Art Unit 1673 | Page 1 of 1 |

**U.S. PATENT DOCUMENTS**

| * | | Document Number Country Code-Number-Kind Code | Date MM-YYYY | Name | Classification |
|---|---|---|---|---|---|
| * | A | US-7,964,580 | 06-2011 | Sofia et al. | 514/51 |
| | B | US- | | | |
| | C | US- | | | |
| | D | US- | | | |
| | E | US- | | | |
| | F | US- | | | |
| | G | US- | | | |
| | H | US- | | | |
| | I | US- | | | |
| | J | US- | | | |
| | K | US- | | | |
| | L | US- | | | |
| | M | US- | | | |

**FOREIGN PATENT DOCUMENTS**

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**NON-PATENT DOCUMENTS**

| * | | Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages) |
|---|---|---|
| | U | |
| | V | |
| | W | |
| | X | |

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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| CONSIGNACION | BANCO DE BOGOTA | 062754387 | 715526420 | 01/07/2015 | 6.398.000.00 |

*** Conceptos Pagados ***

| CANT. | RENTISTICO | CONCEPTO | Vr.UNDITARIO | Vr.CONCEPTO |
|---|---|---|---|---|
| 5 | 50005-01-01 SOLICITUDES | 1607 REIVINDICACION PATENTE UNITARIA ADICIONAL A LAS 10 INIC | 32.000.00 | 160.000.00 |
| | | | | $160.000.00 |

**SON: **CIENTO SESENTA MIL PESOS MONEDA CORRIENTE*****

Responsable: _____
-*****-

Recibo de Caja Aplicado al Expediente No. _____

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Fecha: 2015-07-21 16:50:13 Dep: 2020 DIR.NUEVASCR
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Act: 523 RESPUESTA Folios: 64