PROF. NICOLE POULI (Orcid ID : 0000-0003-3272-759X)

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Novel nucleoside analogues targeting HCV replication through an NS5A-dependent inhibition mechanism

Nikolaos Lougiakis,[a] Efseveia Frakolaki,[b] Panagiota Karmou,[a] Nicole Pouli,*[a] Panagiotis Marakos,[a] Vanesa Madan,[c] Ralf Bartenschlager[c,d,e] and Niki Vassilaki*[b]

[a] *Division of Pharmaceutical Chemistry, Department of Pharmacy, National and Kapodistrian University of Athens, Panepistimiopolis-Zografou, Athens 15771, Greece*

[b] *Molecular Virology Laboratory, Hellenic Pasteur Institute, Vas. Sofias Avenue, 11521, Athens, Greece*

[c] *Department of Infectious Diseases, Molecular Virology, University of Heidelberg, Heidelberg, Germany*

[d] *German Center for Infection Research, Heidelberg University*

[e] *Division of Virus-Associated Carcinogenesis, German Cancer Research Center (DKFZ), Im Neuenheimer Feld 242, 69120 Heidelberg, Germany*

Running title: Tricyclic nucleosides targeting HCV replication

**Address correspondence to:*

N. Pouli, pouli@pharm.uoa.gr, Tel: 0030 2107274185, Fax: 0030 2107274747

N. Vassilaki, nikiv@pasteur.gr Tel: 0030 2106478875, Fax: 0030 2106425038

Abstract

A series of new tricyclic nucleosides were synthesized and evaluated as Hepatitis C virus (HCV) replication inhibitors. Initial screening in a HCV replicon system, derived from a

genotype 1b isolate, identified 9-benzylamino-3-(*β*-D-ribofuranosyl)-3*H*-imidazo[4',5':5,6]pyrido[2,3-*b*]pyrazine (**15d**) as the most potent analogue. Comparative assessment of **15d** activity against HCV full-length viruses or subgenomic replicons derived from genotype 1 - 4 revealed a specificity of the compound for genotypes 1 and 3. Surprisingly, resistance mutations selected against **15d** were mapped to domains II and III of the nonstructural protein 5A (NS5A), but not to the RNA-dependent RNA polymerase residing in NS5B. These results argue that compound **15d** might represent a lead for the development of a novel class of NS5A inhibitors.

Keywords: imidazo[4',5':5,6]pyrido[2,3-*b*]pyrazine; N-ribonucleosides; nucleoside scaffold; antiviral activity; resistance mutations; NS5A domains II, III.

Graphical Abstract

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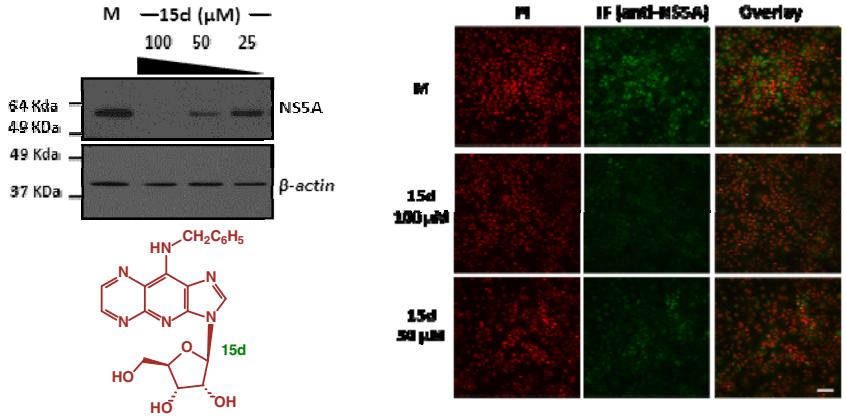

A number of new tricyclic nucleosides were synthesized and their inhibitory potential against HCV was evaluated. The benzylaminoderivative **15d**, reduced HCV replication and possessed selectivity index 4.97 in 1b genotype. Resistance mutation studies were performed, suggesting that this compound could possess a NS5A-dependent mechanism of action, which is unexpected for nucleoside derivatives.

Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease that often leads to liver cirrhosis and hepatocellular carcinoma [1-3]. No vaccine is currently available [4] and treatment aims towards the eradication of the virus. The primary management of HCV infection until recently, consisting of pegylated IFN-α plus ribavirin [5], was only partially effective and associated with numerous side-effects. However, progressive advances in the molecular understanding of the various steps of the viral life cycle [6], along with the introduction of an efficient HCV cell culture system that is based on the JFH1 isolate [7, 8], enabled the development of promising direct acting antivirals (DAAs). Combinations of different approved DAAs, each targeting individual viral proteins, have proven very effective and resulted in multiple IFN-free and largely ribavirin-free regimens, which can safely eliminate HCV in the majority of patients [9-11]. Current drug combinations are orally active and generally well tolerated, but access to diagnosis and therapy remains low on a global scale [12]. Thus, there is a remaining need for novel effective drugs that will avoid the development of resistance, reducing also the high costs of treatment.

HCV belongs to the *Flaviviridae* family [13] and is classified into seven major HCV genotypes and more than one hundred subtypes [14]. The single strand positive-sense RNA genome of HCV is composed of a 5'-untranslated region (UTR), a long open reading frame encoding a polyprotein precursor of about 3,000 amino acids, and a 3′-UTR. The polyprotein precursor is processed into structural proteins (core, E1 and E2), p7 required for assembly and release of virus particles and six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) which form a membrane-associated replicase complex in association with cellular factors [15, 16]. Among the non-structural proteins, the NS3/4A serine-protease complex, the NS5A replicase protein and the NS5B RNA-dependent RNA polymerase (RdRp) are the primary targets for currently available DAAs [15]. In case of NS3/4A, first generation linear peptidomimetic inhibitors were replaced by more potent macrocyclic or

linear second-wave inhibitors, but these still have a limited genotype specificity and a rather low barrier to resistance [17, 18].

RdRp inhibitors fall into two different subclasses, namely nucleos(t)ide (NPIs) and nonnucleoside inhibitors (NNPIs). NNPIs are potent allosteric inhibitors, but with a limited spectrum of activity and a low barrier to resistance. They target five distinct binding pockets located at the thumb (sites 1 and 2) and palm (sites 3, 4 and 5) subdomains of NS5B, which resembles a characteristic human "right hand motif" [19]. On the contrary, NPIs which mimic natural polymerase substrates and bind to the highly conserved active site of the enzyme are characterized by pangenotypic activity and a high barrier to resistance. The uridine nucleotide sofosbuvir (Figure 1) is the most prominent representative of this drug class, and in combination with broadly active NS5A inhibitors results in high cure rates for virtually all genotypes 1-6 [9, 20].

HCV NS5A is a multifunctional RNA-binding phosphoprotein which exists in a basal (56 kDa) and a hyperphosphorylated (58 kDa) form [21, 22]. Available data suggests that the phosphorylation status plays a major role in regulating viral RNA replication versus assembly [23]. NS5A consists of three domains, separated by short low-complexity sequences (LCSs). Domains I (D1) and II (D2) are required for virus genome replication, whereas domain III (D3) is essential for assembly. D1 contributes to binding to lipid droplets, which are crucial for HCV assembly, whereas D3 is involved in interaction with the core protein [24]. D1 can form different homodimers, one of them forming a RNA-binding groove [25]. D2 and D3 of NS5A are intrinsically unfolded and highly flexible, explaining the broad spectrum of protein interactions into which NS5A can engage. For instance, phosphatidylinositol 4-kinase IIIα (PI4KIIIa) interacts with the C-terminal part of D1 [26], cyclophilin A with the C terminus of D2 and with D3 [27, 28] and Src Homology 3 domain-containing proteins (Src family members Lyn and Fyn, mixed lineage kinase 3) with a conserved polyproline (PxxPxR) motif present in low-complexity sequence II (LCSII) [29, 30]. NS5A appears to be phosphorylated at serine and threonine residues by several kinases including casein kinase Iα and II, Mitogen-activated Protein Kinase Kinase 1, AKT (or Protein Kinase B), Polo-like kinase I and cyclic AMP-

dependent protein kinase. Phosphorylation at some of these sites has been reported to affect RNA replication and virus assembly [22].

Clinically approved DAAs against NS5A, as exemplified by daclatasvir (Fig. 1) [31], have been identified by using high content screening approaches. These drugs share as common characteristic two identical peptidomimetic substituents, which suggests binding to a symmetric protein structure.

As part of our involvement in the synthesis and biological evaluation of nucleoside derivatives [32-34], we screened a number of differently substituted compounds with the aim to discover agents with potent anti-HCV activity. In this context, we have identified derivatives of a novel tricyclic nucleoside scaffold, endowed with antiviral activity. It should be noted that the concept of bioactive nucleosides possessing ring-expanded nucleobases has previously been developed [35] and analogously modified derivatives have been found to possess interesting activity against HIV and HCV [36, 37]. We now report the synthesis of new imidazo[4',5':5,6]pyrido[2,3-b]pyrazine ribosides, their evaluation against HCV of genotypes 1-4 and our attempts to clarify their mode of action.

Methods and Materials

Chemistry

Melting points were determined on a Büchi apparatus and are uncorrected. ^{1}H NMR spectra and 2D spectra were recorded on a Bruker Avance III 600 or a Bruker Avance DRX 400 instrument, whereas ^{13}C NMR spectra were recorded on a Bruker Avance III 600 or a Bruker AC 200 spectrometer in deuterated solvents and were referenced to TMS (δ scale). The signals of ^{1}H and ^{13}C spectra were unambiguously assigned by using 2D NMR techniques: ^{1}H^{1}H COSY, NOESY, HMQC, and HMBC. Mass spectra were recorded with a LTQ Orbitrap Discovery instrument, possessing an Ionmax ionization source. Flash chromatography was performed on Merck silica gel 60 (0.040–0.063 mm). Analytical thin layer chromatography (TLC) was carried out on precoated (0.25 mm) Merck silica gel F-254 plates. The purity of all the synthesized compounds was >95% as ascertained by elemental analysis. Elemental

analyses were undertaken using a PerkinElmer PE 240C elemental analyzer (Norwalk, CT, U.S.) and the measured values for C, H, and N were within ±0.4% of the theoretical values.

*N,N'-(3-Nitropyridine-2,6-diyl)diacetamide (**3**)*

To a solution of the diacetamidopyridine **2** (5 g, 25.90 mmol) in concentrated sulfuric acid (37.6 mL) was added dropwise at 0 °C a mixture of sulfuric acid (2.5 mL) and nitric acid (2.5 mL). The mixture was stirred at 0 °C for 20 min and then at room temperature for 25 min. Upon completion of the reaction, the solution was poured into crashed ice and neutralized using ammonium hydroxide (25%) until pH 7. The yellow precipitate was filtered, washed with water and air-dried, to provide the pure nitroderivative **3** (5.5 g, 89%) as a yellow solid, mp 195 °C (EtOAc). ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.11 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 7.99 (d, 1H, J=9 Hz, H-4), 8.38 (d, 1H, J=9 Hz, H-5), 10.68 (s, 1H, NH, D_2O exch.), 10.86 (s, 1H, NH, D_2O exch.). ^{13}C NMR (151 MHz, DMSO-d_6) δ 24.60 (CH_3), 24.69 (CH_3), 109.82 (C-4), 134.72 (C-3), 137.28 (C-5), 143.17 (C-2), 153.94 (C-6), 169.52 (CO), 170.61 (CO). HR-MS (ESI) *m/z*: Calcd for $C_9H_{11}N_4O_4$: $[M1+H]^+$ =239.0775, found 239.0772. Anal. Calcd for $C_9H_{10}N_4O_4$: C, 45.38; H, 4.23; N, 23.52. Found: C, 45.52; H, 4.26; N, 23.34.

*N-(6-Amino-5-nitropyridin-2-yl)acetamide (**4**) and 3-nitropyridine-2,6-diamine (**4a**)*

Hydrochloric acid (36%, 5.4 mL) was added dropwise at 0 °C into a solution of the nitroderivative **3** (2.7 g, 11.34 mmol) in methanol (40 mL) and the mixture was stirred at 0 °C for 40 min and then at room temperature for 1 h. Upon completion of the reaction, the solution was poured into crashed ice and neutralized with ammonium hydroxide (25%) until pH 7. The solvents were vacuum-evaporated, water was added to the residue and it was extracted with EtOAc. The organic extracts were dried over sodium sulfate and evaporated to dryness. The crude mixture was purified by column chromatography (silica gel) using a mixture of dichloromethane / ethyl acetate (from 100/1 up to 100/5, v/v) as the eluent, to provide the nitroderivatives **4** and **4a**.

Data for **4**: Yield 50%. Yellow solid, mp 209 °C (EtOAc). ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.13 (s, 1H, CH_3), 7.44 (d, 1H, J=9 Hz, H-4), 7.75 (brs, 2H, NH_2, D_2O exch.), 8.36 (d, 1H,

J=9 Hz, H-5), 10.64 (s, 1H, NH, D_2O exch.). ^{13}C NMR (151 MHz, DMSO-d_6) δ 24.73 (CH_3), 103.48 (C-4), 123.23 (C-3), 137.96 (C-5), 153.93 (C-2), 156.56 (C-6), 170.77 (CO). HR-MS (ESI) *m/z*: Calcd for $C_7H_9N_4O_3$: $[M1+H]^+$ =197.0669, found 197.0661. Anal. Calcd for $C_7H_8N_4O_3$: C, 42.86; H, 4.11; N, 28.56. Found: C, 42.99; H, 4.14; N, 28.35.

Data for **4a**: Yield 3%. Yellow solid, mp 240 °C (EtOAc). ^1H NMR (600 MHz, DMSO-d_6) δ 5.92 (d, 1H, J=9 Hz, H-4), 7.27 (brs, 1H, NH, D_2O exch.), 7.59 (brs, 1H, NH, D_2O exch.), 7.97 (d, 1H, J=9 Hz, H-5), 8.04 (brs, 2H, NH_2, D_2O exch.). ^{13}C NMR (151 MHz, DMSO-d_6) δ 101.86 (C-4), 117.77 (C-3), 135.79 (C-5), 156.21 (C-2), 162.80 (C-6). HR-MS (ESI) *m/z*: Calcd for $C_5H_7N_4O_2$: $[M1+H]^+$ =155.0564, found 155.0559. Anal. Calcd for $C_5H_6N_4O_2$: C, 38.96; H, 3.92; N, 36.35. Found: C, 38.78; H, 3.87; N, 36.51.

*N-(Pyrido[2,3-b]pyrazin-6-yl)acetamide (**5**)*

A solution of the nitroderivative **4** (500 mg, 2.55 mmol) in absolute ethanol (30 mL) was hydrogenated in the presence of 10% Pd/C (60 mg) under a pressure of 50 psi at room temperature for 4h. The solution was then filtered through a celite pad and the filtrate was evaporated to dryness. The resulting diamine was dissolved in ethanol (30 mL), glyoxal (40% w/w aq. solution, 0.41 g, 2.8 mmol) was added to the solution and the mixture was refluxed for 90 min. The solvents were then evaporated and the residue was purified by column chromatography (silica gel) using ethyl acetate as the eluent, to provide the compound **5** as a beige solid (0.54 g, 96%). mp 240 °C (EtOAc). ^1H NMR (600 MHz, DMSO-d_6) δ 2.19 (s, 3H, CH_3), 8.49 (d, 1H, J=9 Hz, H-8), 8.62 (d, 1H, J=9 Hz, H-7), 8.90 (d, 1H, J=2 Hz, H-2), 9.03 (d, 1H, J=2 Hz, H-3), 11.28 (s, 1H, NH, D_2Oexch.). ^{13}C NMR (151 MHz, DMSO-d_6) δ 24.37 (CH_3), 119.09 (C-7), 135.97 (C-8a), 140.04 (C-8), 144.41 (C-2), 148.16 (C-3), 150.98 (C-4a), 154.73 (C-6), 170.67 (CO). HR-MS (ESI) *m/z*: Calcd for $C_9H_9N_4O$: $[M1+H]^+$ =189.0771, found 189.0767. Anal. Calcd for $C_9H_8N_4O$: C, 57.44; H, 4.29; N, 29.77. Found: C, 57.66; H, 4.36; N, 29.59.

*7-Nitropyrido[2,3-b]pyrazin-6-amine (**6**)*

A mixture of fuming nitric acid (0.4 mL) and acetic anhydride (1.6 mL) was added dropwise into a solution of the acetamide **5** (250 mg, 1.33 mmol) in acetic anhydride (6 mL) at -20 °C,

and the mixture was stirred at this temperature for 90 min. The solution was then poured into crashed ice, extracted with ethyl acetate, the organic extracts were dried over sodium sulfate and evaporated to dryness. The crude mixture was purified by column chromatography (silica gel) using a mixture of cyclohexane / ethyl acetate (4/6, v/v) as the eluent, to provide the nitrocompound **6** (20 mg, 6%) as an orange colored solid. mp > 300 °C (EtOAc). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.84 (brs, 1H, NH_2, D_2O exch.), 8.14 (brs, 1H, NH_2, D_2O exch.), 8.73 (d, 1H, J=1.9 Hz, H-2), 8.95 (d, 1H, J=1.9 Hz, H-3), 8.99 (s, 1H, H-8). ^{13}C NMR (151 MHz, DMSO-d_6) δ 131.28 (C-8a), 134.10 (C-7), 137.23 (C-8), 142.47 (C-2), 150.04 (C-6), 151.92 (C-3), 153.79 (C-4a). HR-MS (ESI) *m/z*: Calcd for $C_7H_6N_5O_2$: $[M1+H]^+$ =192.0516, found 192.0507. Anal. Calcd for $C_7H_5N_5O_2$: C, 43.98; H, 2.64; N, 36.64. Found: C, 44.11; H, 2.69; N, 36.53.

*N-(3H-imidazo[4,5-b]pyridin-5-yl)acetamide (**7**)*

The nitroderivative **4** (1.2 g, 6.12 mmol) was hydrogenated according to the method reported for the preparation of compound **5**. The resulting diamine was then dissolved in triethylorthoformate (6 mL) followed by dropwise addition of hydrochloric acid (36%, 0.1 mL) and the mixture was stirred at room temperature for 30 h. Upon completion of the reaction, *n*-pentane (50 mL) was added and the precipitate was filtered, washed with *n*-pentane and air-dried. The solid was dissolved in methanol, sodium bicarbonate (0.5 g) was added and the mixture was stirred at room temperature for 1h. The solvent was evaporated and the crude product was purified by column chromatography (silica gel) using a mixture of dichloromethane / methanol (100/1, v/v) as the eluent, to provide the acetamide **7** (1 g, 93%). mp 212-3 °C (EtOAc). ^{1}H NMR (600 MHz, DMSO-d_6) δ 2.10 (s, 3H, CH_3), 7.99 (d, 1H, J=8.5Hz, H-7), 8.02 (d, 1H, J=8.5Hz, H-6), 8.33 (s, 1H, H-2), 10.46 (s, 1H, NHCO, D_2O exch.), 12.80 (s, 1H, NH-3, D_2O exch.). ^{13}C NMR (151 MHz, DMSO-d_6) δ 24.43 (CH_3), 109.94 (C-6), 125.78 (C-7), 127.74 (C-7a), 143.65 (C-2), 148.17 (C-5), 149.49 (C-3a), 169.28 (CO). HR-MS (ESI) *m/z*: Calcd for $C_8H_9N_4O$: $[M1+H]^+$ =177.0771, found 177.0765. Anal. Calcd for $C_8H_8N_4O$: C, 54.54; H, 4.58; N, 31.80. Found: C, 54.73; H, 4.64; N, 31.61.

*6-Nitro-3H-imidazo[4,5-b]pyridin-5-amine (**8**)*

This compound was prepared following a methodology analogous to that reported for **3**. The product was purified by column chromatography (silica gel) using a mixture of dichloromethane / methanol (92/8, v/v) as the eluent, to provide the nitroderivative **8** (0.69 g, 68%) as an orange colored solid. mp >300 °C (EtOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.75 (s, 2H, NH_2, D_2O exch.), 8.36 (s, 1H, H-2), 8.60 (s, 1H, H-7). ^{13}C NMR (151 MHz, DMSO-d_6) δ 125.15 (C-7), 126.92 (C-6), 127.31 (C-7a), 147.11 (C-2), 152.71 (C-5), 154.09 (C-3a). HR-MS (ESI) *m/z*: Calcd for $C_6H_6N_5O_2$: $[M1+H]^+$ =180.0516, found 180.0509. Anal. Calcd for $C_6H_5N_5O_2$: C, 40.23; H, 2.81; N, 39.10. Found: C, 40.46; H, 2.88; N, 38.94.

*3H-Imidazo[4',5':5,6]pyrido[2,3-b]pyrazine (**9**)*

This compound was prepared from the nitroderivative **8**, following a methodology analogous to that reported for **3**. The hydrogenation lasted 2 days and the product was obtained upon repeated recrystallizations with methanol in 56% yield. mp >300 °C (MeOH). A small quantity of the compound was purified by column chromatography (silica gel) for analytical reasons, using a mixture of ethyl acetate / methanol (9/1, v/v) as the eluent. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.74 (s, 1H, H-2), 8.94 (s, 1H, H-9), 9.00 (s, 1H, H-7), 9.08 (s, 1H, H-6). ^{13}C NMR (151 MHz, DMSO-d_6) δ 127.31 (C-9a), 132.93 (C-8a), 135.16 (C-9), 144.46 (C-7), 146.57 (C-6), 147.47 (C-2), 151.68 (C-4a), 153.11 (C-3a). HR-MS (ESI) *m/z*: Calcd for $C_8H_6N_5$: $[M1+H]^+$ =172.0618, found 172.0611. Anal. Calcd for $C_8H_5N_5$: C, 56.14; H, 2.94; N, 40.92. Found: C, 56.27; H, 2.99; N, 40.69.

*3-(2,3,5-Tri-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazine (**10**) and 1-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-1H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazine (**11**)*

N,O-Bis(trimethylsilyl)acetamide (1.67 mL, 6.81 mmol) was added under argon to a suspension of compound **9** (1 g, 5.84 mmol) in anhydrous acetonitrile (35 mL) and the mixture was heated at 80 °C for 45 min. Upon cooling at room temperature, tetra-*O*-acetyl-*β*-D-ribofuranose (2 g, 6.28 mmol) was added, followed by dropwise addition of trimethylsilyl trifluoromethanesulfonate (1.06 mL, 5.84 mmol) at 0 °C and the mixture was refluxed for 3 hours. The solvents were then evaporated, the crude material was diluted with ethyl acetate and extracted with a saturated $NaHCO_3$ solution. The aqueous layer was extracted twice

more with ethyl acetate, the combined organic extracts were dried over sodium sulfate and evaporated to dryness. The crude mixture was purified by column chromatography, using initially a mixture of cyclohexane / ethyl acetate (from 2/8 up to 0/10, v/v) and then a mixture of ethyl acetate / methanol (from 100/0 up to 95/5, v/v) as the eluent, providing 1.03 g of **10** (as a mixture with its corresponding *α*-anomer, ratio 7 : 1, as indicated by NMR), and 0.35 g of **11** (as a mixture with its corresponding *α*-anomer, ratio 7 : 1, as indicated by NMR). The mixture of **10** with its *α*-anomer was repurified by column chromatography using a mixture of dichloromethane / ethyl acetate (6/4, v/v) as the eluent and the target *β*-anomer could be isolated pure, as a pale yellow gum (0.77 g, 31%). On the other hand, the mixture of **11** with its *α*-anomer was repurified by column chromatography using a mixture of dichloromethane / methanol (100/1, v/v) as the eluent and the target *β*-anomer was isolated pure, as a pale yellow solid (0.17 g, 7%).

Data for **10**: ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.03 (s, 3H, CH_3), 2.15 (s, 3H, CH_3), 2.16 (s, 3H, CH_3), 4.45 (m, 2H, H-5'), 4.50 (m, 1H, H-4'), 5.65 (m, 1H, H-3'), 5.90 (m, 1H, H-2'), 6.65 (d, 1H, J=5.8 Hz, H-1'), 7.72 (s, 1H, H-2), 8.85 (s, 1H, H-9), 8.96 (d, 1H, J=1.6 Hz, H-7), 9.05 (d, 1H, J=1.6 Hz, H-6). ^{13}C NMR (151 MHz, $CDCl_3$) δ 20.40 (CH_3), 20.57 (CH_3), 20.85 (CH_3), 63.19 (C-5'), 70.77 (C-3'), 73.30 (C-2'), 80.35 (C-4'), 85.50 (C-1'), 128.96 (C-9), 136.53 (C-8a), 138.19 (C-9a), 144.21 (C-7), 146.77 (C-6), 148.17 (C-2), 148.36 (C-4a), 149.98 (C-3a), 169.50 (CO), 169.67 (CO), 170.29 (CO). HR-MS (ESI) *m/z*: Calcd for $C_{19}H_{20}N_5O_7$: $[M1+H]^+$ =430.1357, found 430.1350. Anal. Calcd for $C_{19}H_{19}N_5O_7$: C, 53.15; H, 4.46; N, 16.31. Found: C, 53.02; H, 4.43; N, 16.46.

Data for **11**: mp 92-3 °C ($CHCl_3$/Et_2O). ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.09 (s, 3H, CH_3), 2.18 (s, 3H, CH_3), 2.32 (s, 3H, CH_3), 4.44 (dd, 1H, J=13.2 Hz, J= 2.9 Hz, H-5'), 4.52-4.59 (m, 2H, H-4', H-5'), 5.48 (m, 1H, H-3'), 5.66 (m, 1H, H-2'), 6.24 (d, 1H, J= 5.7 Hz, H-1'), 8.79 (s, 1H, H-9), 8.81 (s, 1H, H-2), 8.94 (s, 1H, J=1.4 Hz, H-7), 9.11 (s, 1H, J=1.4 Hz, H-6). ^{13}C NMR (50 MHz, $CDCl_3$) δ 20.46 (CH_3), 20.65 (CH_3), 21.11 (CH_3), 62.93 (C-5'), 70.17 (C-3'), 72.79 (C-2'), 80.97 (C-4'), 88.24 (C-1'), 119.48 (C-9), 127.36 (C-9a), 135.76 (C-8a), 144.99 (C-7), 146.95 (C-6), 148.64 (C-4a), 150.98 (C-2), 159.88 (C-3a), 169.44 (CO), 169.69 (CO), 170.46

(CO). HR-MS (ESI) *m/z*: Calcd for $C_{19}H_{20}N_5O_7$: $[M1+H]^+$ =430.1357, found 430.1352. Anal. Calcd for $C_{19}H_{19}N_5O_7$: C, 53.15; H, 4.46; N, 16.31. Found: C, 53.32; H, 4.53; N, 16.16.

*General procedure for the synthesis of nucleosides **12** and **13***

Compound **10** or **11** (130 mg, 0.3 mmol) was dissolved in a saturated solution of ammonia in methanol (15 mL) and the resulting mixture was stirred at room temperature for 6 h. The solvent was vacuum-evaporated and the residue was purified by column chromatography.

*3-(β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazine (**12**)*

Purification was effected using a mixture of dichloromethane / methanol (96/4, v/v) as the eluent to provide the nucleoside **12** as a white solid. Yield 87%. mp 227-8 °C (MeOH). $[\alpha]_D$ -31.76° (*c*=0.296, DMSO). ^{1}H NMR (400 MHz, DMSO-d_6) δ 3.63 (m, 1H, H-5'), 3.76 (m, 1H, H-5'), 4.03 (m, 1H, H-4'), 4.26 (m, 1H, H-3'), 4.76 (m, 1H, H-2'), 5.22 (t, 1H, J=5.7 Hz, OH-5', D_2O exch.), 5.29 (d, 1H, J=4.9 Hz, OH-3', D_2O exch.), 5.58 (d, 1H, J=5.9 Hz, OH-2', D_2O exch.), 6.23 (d, 1H, J=5.7 Hz, H-1'), 8.88 (s, 1H, H-9), 9.04 (d, 1H, J=1.7 Hz, H-7), 9.12 (d, 1H, J=1.7 Hz, H-6), 9.18 (s, 1H, H-2). ^{13}C NMR (50 MHz, DMSO-d_6) δ 61.81 (C-5'), 70.85 (C-3'), 73.90 (C-2'), 86.16 (C-4'), 88.14 (C-1'), 127.95 (C-9), 135.93 (C-8a), 138.78 (C-9a), 144.81 (C-7), 147.47 (C-6), 147.83 (C-4a), 150.59 (C-3a), 151.36 (C-2). HR-MS (ESI) *m/z*: Calcd for $C_{13}H_{14}N_5O_4$: $[M1+H]^+$ =304.1040, found 304.1032. Anal. Calcd for $C_{13}H_{13}N_5O_4$: C, 51.49; H, 4.32; N, 23.09. Found: C, 51.68; H, 4.39; N, 22.91.

*1-(β-D-ribofuranosyl)-1H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazine (**13**)*

Purification was effected using a mixture of ethyl acetate / methanol (98/2, v/v) as the eluent to provide the nucleoside **13** as a white solid. Yield 82%. mp 218-9 $°C_{(dec.)}$ (MeOH). $[\alpha]_D$ -59.41° (*c*=0.239, DMSO). ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.69-3.77 (m, 2H, H-5'), 4.08 (m, 1H, H-4'), 4.19 (m, 1H, H-3'), 4.49 (m, 1H, H-2'), 5.31 (d, 1H, J=4.6 Hz, OH-3', D_2O exch.), 5.34 (t, 1H, J=4.9 Hz, OH-5', D_2O exch.), 5.61 (d, 1H, J=6.3 Hz, OH-2', D_2O exch.), 6.09 (d, 1H, J=6.3 Hz, H-1'), 9.03 (d, 1H, J=1.6 Hz, H-7), 9.06 (s, 1H, H-9), 9.12 (d, 1H, J=1.6 Hz, H-6), 9.23 (s, 1H, H-2). ^{13}C NMR (50 MHz, DMSO-d_6) δ 61.10 (C-5'), 70.12 (C-3'), 73.62 (C-2'), 86.08 (C-4'), 89.83 (C-1'), 119.60 (C-9), 127.58 (C-9a), 134.79 (C-8a), 144.78 (C-7), 146.67 (C-6), 147.43 (C-4a), 152.93 (C-2), 159.69 (C-3a). HR-MS (ESI) *m/z*: Calcd for $C_{13}H_{14}N_5O_4$:

$[M1+H]^+$ =304.1040, found 304.1034. Anal. Calcd for $C_{13}H_{13}N_5O_4$: C, 51.49; H, 4.32; N, 23.09. Found: C, 51.30; H, 4.28; N, 23.28.

*9-Chloro-3-(2,3,5-tri-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazine (**14**)*

N-Chlorosuccinimide (100 mg, 0.75 mmol) was added under argon in a solution of the derivative **10** (230 mg, 0.54 mmol) in anhydrous THF (10 mL) and the mixture was stirred at room temperature for 24 h. The solvent was then removed in vacuo, the residue was extracted with ethyl acetate and water, the organic extracts were dried over sodium sulfate and evaporated to dryness. The crude product was purified by column chromatography (silica gel) using a mixture of cyclohexane / ethyl acetate (6/4, v/v) as the eluent, to provide the chloroderivative **14** (150 mg, 60%) as a pale yellow gum. ^{1}H NMR (600 MHz, $CDCl_3$) δ 2.03 (s, 3H, CH_3), 2.16 (s, 6H, 2 x CH_3), 4.45 (m, 2H, H-5'), 4.51 (m, 1H, H-4'), 5.63 (m, 1H, H-3'), 5.89 (m, 1H, H-2'), 6.60 (d, 1H, J=5.8 Hz, H-1'), 8.67 (s, 1H, H-2), 9.04 (d, 1H, J=1.6 Hz, H-7), 9.10 (d, 1H, J=1.6 Hz, H-6). ^{13}C NMR (151 MHz, $CDCl_3$) δ 20.31 (CH_3), 20.49 (CH_3), 20.75 (CH_3), 63.31 (C-5'), 70.75 (C-3'), 73.28 (C-2'), 80.43 (C-4'), 85.61 (C-1'), 133.60 (C-9), 134.30 (C-8a), 136.16 (C-9a), 143.99 (C-7), 147.22 (C-6), 148.26 (C-2), 148.64 (C-4a), 149.45 (C-3a), 169.40 (CO), 169.58 (CO), 170.20 (CO). HR-MS (ESI) *m/z*: Calcd for $C_{19}H_{19}ClN_5O_7$: $[M1+H]^+$ =464.0968, found 464.0963. Anal. Calcd for $C_{19}H_{18}ClN_5O_7$: C, 49.20; H, 3.91; N, 15.10. Found: C, 49.09; H, 3.88; N, 15.26.

*General procedure for the synthesis of the target compounds **15a-e***

2-Dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl (X-phos, 6.2 mg, 0.013 mmol), tris(dibenzylideneacetone)dipalladium(0) (12 mg, 0.013 mmol) and the appropriate amine (1.3 mmol) were added under argon in a solution of the chloroderivative **14** (120 mg, 0.26 mmol) in anhydrous dioxane (3 mL) and the resulting mixture was heated at 90 °C for 20 h. The solvents were then vacuum-evaporated and the crude mixture was treated with a saturated solution of ammonia in methanol (10 mL) for 18 h at room temperature. The solvent was evaporated and the residue was purified by column chromatography to provide the aminosubstituted tricyclic nucleosides **15a-f**.

*N-(Butyl)-3-(β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazin-9-amine (**15a**)*

This compound was prepared according to the general procedure described above, upon reaction of **14** with *n*-butylamine. Purification was effected using a mixture of chloroform / methanol (95/5, v/v) as the eluent. Yield 39%. mp 227-8 °C (MeOH). $[\alpha]_D$ -10.77° (*c*=0.130, MeOH). ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.92 (t, 3H, J=7.4 Hz, CH_3), 1.40 (m, 2H, CH_2CH$_3$), 1.73 (m, 2H, CH_2CH$_2$CH$_3$), 3.59 (m, 1H, H-5'), 3.72 (m, 1H, H-5'), 4.00 (m, 1H, H-4'), 4.13 (m, 2H, NHCH_2CH$_2$), 4.20 (m, 1H, H-3'), 4.74 (m, 1H, H-2'), 5.18 (d, 1H, J=4.6 Hz, OH-3', D_2O exch.), 5.44 (d, 1H, J=6.2 Hz, OH-2', D_2O exch.), 5.57 (m, 1H, OH-5', D_2O exch.), 6.05 (d, 1H, J=6.3 Hz, H-1'), 8.20 (t, 1H, J=6.6 Hz, NH, D_2O exch.), 8.54 (s, 1H, H-2), 8.66 (d, 1H, J=1.7 Hz, H-7), 8.94 (d, 1H, J=1.7 Hz, H-6). ^{13}C NMR (50 MHz, DMSO-d_6) δ 13.81 (CH_3), 19.43 (*C*H$_2$CH$_3$), 32.28 (*C*H$_2$CH$_2$CH$_3$), 43.47 (NH*C*H$_2$), 61.75 (C-5'), 70.72 (C-3'), 73.00 (C-2'), 85.86 (C-4'), 87.67 (C-1'), 119.61 (C-9a), 125.98 (C-8a), 138.38 (C-7), 142.64 (C-2), 143.38 (C-9), 146.96 (C-6), 148.54 (C-4a), 150.89 (C-3a). HR-MS (ESI) *m/z*: Calcd for $C_{17}H_{23}N_6O_4$: $[M1+H]^+$ =375.1775, found 375.1771. Anal. Calcd for $C_{17}H_{22}N_6O_4$: C, 54.54; H, 5.92; N, 22.45. Found: C, 54.38; H, 5.95; N, 22.56.

*N-(3-Methylbutyl)-3-(β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazin-9-amine (**15b**)*

This compound was prepared according to the general procedure described above, upon reaction of **14** with 3-methylbutylamine. Purification was effected using a mixture of dichloromethane / methanol (100/4, v/v) as the eluent. Yield 60%. mp 218 °C (MeOH/THF). $[\alpha]_D$ -37.04° (*c*=0.189, DMSO). ^{1}H NMR (600 MHz, DMSO-d_6) δ 0.93 (d, 6H, J=6.5 Hz, 2 x CH_3), 1.62 (q, 2H, J=7.1 Hz, CH_2CH(CH$_3$)$_2$), 1.70 (m, 1H, CH$_2$C*H*(CH$_3$)$_2$), 3.59 (m, 1H, H-5'), 3.72 (m, 1H, H-5'), 4.00 (m, 1H, H-4'), 4.12-4.22 (m, 3H, H-3', HNCH_2CH$_2$), 4.73 (m, 1H, H-2'), 5.21 (d, 1H, J=4.5 Hz, OH-3', D_2O exch.), 5.47 (d, 1H, J=6.3 Hz, OH-2', D_2O exch.), 5.62 (m, 1H, OH-5', D_2O exch.), 6.04 (d, 1H, J=6.3 Hz, H-1'), 8.15 (t, 1H, J=6.4 Hz, NH, D_2O exch.), 8.53 (s, 1H, H-2), 8.66 (d, 1H, J=1.6 Hz, H-7), 8.93 (d, 1H, J=1.6 Hz, H-6). ^{13}C NMR (151 MHz, DMSO-d_6) δ 22.70 (CH(*C*H$_3$)$_2$), 25.49 (*C*H$_2$CH(CH$_3$)$_2$), 40.06 (CH$_2$*C*H(CH$_3$)$_2$), 42.28 (HN*C*H$_2$CH$_2$), 61.87 (C-5'), 70.83 (C-3'), 73.12 (C-2'), 85.98 (C-4'), 87.79 (C-1'),

119.70 (C-9a), 126.06 (C-8a), 138.56 (C-7), 142.74 (C-2), 143.46 (C-9), 147.11 (C-6), 148.63 (C-4a), 150.97 (C-3a). HR-MS (ESI) *m/z*: Calcd for $C_{18}H_{25}N_6O_4$: $[M1+H]^+$ =389.1932, found 389.1929. Anal. Calcd for $C_{18}H_{24}N_6O_4$: C, 55.66; H, 6.23; N, 21.64. Found: C, 55.75; H, 6.26; N, 21.51.

*N-(3-Methylbut-2-en-1-yl)-3-(β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazin-9-amine (**15c**)*

This compound was prepared according to the general procedure described above, upon reaction of **14** with 3-methylbut-2-en-1-amine. Purification was effected using a mixture of dichloromethane / methanol (100/4, v/v) as the eluent. Yield 45%. mp 209 °C (MeOH). $[\alpha]_D$ -39.45° (*c*=0.256, DMSO). ^{1}H NMR (600 MHz, DMSO-d_6) δ 1.67 (s, 3H,CH_3), 1.76 (s, 3H,CH_3), 3.59 (m, 1H, H-5'), 3.72 (m, 1H, H-5'), 4.01 (m, 1H, H-4'), 4.20 (m, 1H, H-3'), 4.70-4.79 (m, 3H, H-2', HNCH_2CH), 5.18 (d, 1H, J=4.6 Hz, OH-3', D_2O exch.), 5.43-5.49 (m, 2H, OH-2', D_2O exch., CH_2CH=C$(CH_3)_2$), 5.56 (m, 1H, OH-5', D_2O exch.), 6.05 (d, 1H, J=6.2 Hz, H-1'), 8.12 (t, 1H, J=6.4 Hz, NH, D_2O exch.), 8.56 (s, 1H, H-2), 8.66 (d, 1H, J=1.7 Hz, H-7), 8.94 (d, 1H, J=1.7 Hz, H-6). ^{13}C NMR (151 MHz, DMSO-d_6) δ 18.00 (CH_3), 25.47 (CH_3), 41.73 (HNCH_2CH), 61.74 (C-5'), 70.71 (C-3'), 73.01 (C-2'), 85.85 (C-4'), 87.65 (C-1'), 119.75 (C-9a), 122.31 (CH=C$(CH_3)_2$), 125.91 (C-8a), 133.92 (CH=C$(CH_3)_2$), 138.45 (C-7), 142.72 (C-2), 143.00 (C-9), 146.99 (C-6), 148.51 (C-4a), 150.86 (C-3a). HR-MS (ESI) *m/z*: Calcd for $C_{18}H_{23}N_6O_4$: $[M1+H]^+$ =387.1775, found 387.1770. Anal. Calcd for $C_{18}H_{22}N_6O_4$: C, 55.95; H, 5.74; N, 21.75. Found: C, 56.04; H, 5.78; N, 21.63.

*N-(Benzyl)-3-(β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazin-9-amine (**15d**)*

This compound was prepared according to the general procedure described above, upon reaction of **14** with benzylamine. Purification was effected using a mixture of dichloromethane / methanol (100/4, v/v) as the eluent. Yield 52%. mp 227-8 °C (MeOH). $[\alpha]_D$-48.33° (*c*=0.269, DMSO). ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.58 (m, 1H, H-5'), 3.71 (m, 1H, H-5'), 3.99 (m, 1H, H-4'), 4.19 (m, 1H, H-3'), 4.73 (m, 1H, H-2'), 5.18 (d, 1H, J=4.6 Hz, OH-3', D_2O exch.), 5.42 (m, 2H, C$H_2C_6H_5$), 5.45 (d, 1H, J=6.2 Hz, OH-2', D_2O exch.), 5.52 (m, 1H, OH-5', D_2O exch.), 6.05 (d, 1H, J=6.3 Hz, H-1'), 7.20 (t, 1H, J=7.4 Hz, H-4''), 7.28 (t,

2H, J=7.5 Hz, H-3'', H-5''), 7.43 (d, 2H, J=7.5 Hz, H-2'', H-6''), 8.56 (s, 1H, H-2), 8.68-8.72 (m, 2H, H-7, NH), 8.97 (d, 1H, J=1.7 Hz, H-6). ^{13}C NMR (50 MHz, DMSO-d_6) δ 47.17 ($CH_2C_6H_5$), 62.19 (C-5'), 71.15 (C-3'), 73.46 (C-2'), 86.30 (C-4'), 88.05 (C-1'), 120.30 (C-9a), 126.35 (C-8a), 127.20 (C-4''), 127.66 (C-3'', C-5''), 128.74 (C-2'', C-6''), 139.12 (C-7), 140.79 (C-1'), 143.24 (C-2), 143.62 (C-9), 147.54 (C-6), 149.94 (C-4a), 151.33 (C-3a). HR-MS (ESI) *m/z*: Calcd for $C_{20}H_{21}N_6O_4$: $[M1+H]^+$ =409.1619, found 409.1613. Anal. Calcd for $C_{20}H_{20}N_6O_4$: C, 58.82; H, 4.94; N, 20.58. Found: C, 58.97; H, 4.97; N, 20.41.

*N-(Furan-2-ylmethylen)-3-(β-D-ribofuranosyl)-3H-imidazo[4',5':5,6]pyrido[2,3-b]pyrazin-9-amine (**15e**)*

This compound was prepared according to the general procedure described above, upon reaction of **14** with 2-aminomethylfuran. Purification was effected using a mixture of dichloromethane / methanol (100/4, v/v) as the eluent. Yield 45%. mp 212-3 °C (MeOH/THF). $[\alpha]_D$ -55.74° (*c*=0.296, DMSO). ^{1}H NMR (600 MHz, DMSO-d_6) δ 3.59 (m, 1H, H-5'), 3.72 (m, 1H, H-5'), 4.00 (m, 1H, H-4'), 4.21 (m, 1H, H-3'), 4.74 (m, 1H, H-2'), 5.19 (d, 1H, J=4.6 Hz, OH-3', D_2O exch.), 5.41 (m, 2H, HNCH_2), 5.46 (d, 1H, J=6.2 Hz, OH-2', D_2O exch.), 5.51 (m, 1H, OH-5', D_2O exch.), 6.07 (d, 1H, J=6.2 Hz, H-1'), 6.29 (m, 1H, H-3''), 6.34 (m, 1H, H-4''), 7.55 (m, 1H, H-5''), 8.47 (t, 1H, J=6.7 Hz, NH, D_2O exch.), 8.61 (s, 1H, H-2), 8.70 (d, 1H, J=1.7 Hz, H-7), 8.97 (d, 1H, J=1.7 Hz, H-6). ^{13}C NMR (50 MHz, DMSO-d_6) δ 40.63 (HNCH_2-furan), 61.70 (C-5'), 70.69 (C-3'), 73.03 (C-2'), 85.83 (C-4'), 87.57 (C-1'), 106.93 (C-3''), 110.40 (C-4''), 120.07 (C-9a), 125.82 (C-8a), 138.85 (C-7), 142.14 (C-5''), 142.77 (C-2), 143.01 (C-9), 147.11 (C-6), 148.36 (C-4a), 150.85 (C-3a), 153.01 (C-2''). HR-MS (ESI) *m/z*: Calcd for $C_{18}H_{19}N_6O_5$: $[M1+H]^+$ =399.1411, found 399.1404. Anal. Calcd for $C_{18}H_{18}N_6O_5$: C, 54.27; H, 4.55; N, 21.10. Found: C, 54.13; H, 4.48; N, 21.33.

Biological evaluation

Cell lines, viruses and plasmid constructs

The replication permissive Huh7-Lunet [38] cells and the replication and infection permissive Huh7.5 [39] cells were cultured in high glucose (25 mM) Dulbecco's modified minimal

essential medium (Invitrogen), supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 100 U/mL penicillin, 100 µg/mL streptomycin and 10% (v/v) fetal calf serum (referred to as complete DMEM).

Huh5-2 [40] and Huh7-JFH1 [41] stable cell lines harbor the subgenomic HCV reporter replicons I_{389}luc-ubi-neo/NS3-3'/Con1/5.1 (genotype 1b, Con1 strain) and I_{389}luc-ubi-neo/NS3-3'_dg_JFH1 (genotype 2a, JFH1 strain), respectively. Specifically, they carry the HCV 5' UTR with the internal ribosome entry site (IRES) directing the expression of a firefly luciferase-ubiquitin-neomycin phosphotransferase fusion cassette (luc-ubi-neo), followed by an IRES from encephalomyocarditis virus (EMCV) that directs translation of the HCV NS3 to NS5B region, and the HCV 3' UTR. The expression of luc-ubi-neo gene enables the concurrent selection of neomycin/G418-resistant cells expressing the HCV RNA and the assessment of RNA replication levels by measuring the activity of the reporter protein firefly luciferase (F-Luc). Huh 5-2 and Huh7-JFH1 cells were grown in complete DMEM supplemented with 500 µg/mL G418 or 1mg/mL respectively.

Bicistronic replicon plasmids S52-SG (Feo) (AII) and ED43-SG (Feo)(VYG) (kindly provided by C.M. Rice, The Rockefeller University, NY) have been described previously [42] and encode a chimeric gene of F-Luc protein fused in frame with neomycin phosphotransferase (Feo), under the HCV IRES control, and the subgenomic sequences of HCV genotype 3a (strain S52) and 4a (strain ED43), respectively, under the EMCV IRES control. Stable cell lines were generated by transfecting Huh7.5 cells with the above replicon RNAs and pooling colonies after selection with 750 µg/ml G418 for Huh7.5-3a and 350 µg/mL G418 for Huh7.5-4a. After selection, cells were mentained in complete DMEM supplemented with the same G418 concentrations.

Plasmids pH77S.3/GLuc2A [43] and pHCV-N.2/GLuc2A [44] (kindly provided by S.M. Lemon, University of North Carolina, USA) encode full-length reporter HCV genome sequences of genotype 1a (strain H77) and 1b (strain HCV-N), respectively, with adaptive mutations. The *Gaussia* luciferase (G-Luc) gene followed by the FMDV2A sequence is included between HCV p7 and NS2.

All amino acid and nucleotide numbers refer to the Con1 genome (GenBank accession no. AJ238799).

***In vitro* transcription**

Full-length and bicistronic HCV constructs were linearized with XbaI and used for in vitro transcription as described previously [45].

Transfection with *in vitro* transcribed RNA

Electroporation with full-length viral RNAs into Huh7-Lunet cells and bicistronic HCV RNAs into Huh7.5 cells was performed as described elsewhere [46].

Anti-HCV assay

Anti-HCV assay in replicon cells was performed by seeding $1x10^4$ cells per well in a 96-well flat bottom plate cultured in 200 µL complete DMEM supplemented with G418. Following 24 h incubation at 37°C (5% CO_2), medium was removed and 2-fold serial dilutions (without G418) of the test compounds in complete DMEM were added, in a total volume of 100 µL. After 3 days of incubation at 37°C, cells were lysed and F-Luc activity was measured.

Anti-HCV assay for full-length viral constructs was performed after electroporation of Huh7-Lunet cells with HCV RNA. Cells were resuspended in complete DMEM and seeded in tetraplicates on 48-well plates. 24 h after electroporation, medium was removed and 2-fold serial dilutions of the test compounds in complete DMEM were added, in a total volume of 200 µL. 24 and 48 h after compound addition, medium was exchanged and at 72 h cell supernatants were collected and G-Luc activity was measured. In addition, cells were lysed for total protein quantification.

Relative luminescence units (RLU) were expressed as percentage of the respective values from DMSO-treated control cells. The half maximal effective concentration (EC_{50}) was defined as the concentration of compound that reduced the luciferase signal by 50%.

EC_{50} values were determined by nonlinear regression analysis after converting the drug concentrations into log-X using Prism 5.0 software (GraphPad Software Inc.).

Luciferase and Bradford assays

F-Luc activity in cell lysates was measured using the respective chemiluminescent assay kit (Promega), as manufacturer recommended. G-Luc activity in cell supernatants was measured using 12 µM Coelenterazine (Promega) in assay buffer (50 mM potassium phosphate, pH 7.4, 500 mM NaCl, 1 mM EDTA). Measurements were performed with a GloMax 20/20 single tube luminometer (Promega) for 10 s. F-Luc and G-Luc activities were normalized to the intracellular total protein amount, determined using the Bradford assay reagent (Pierce).

Cytotoxicity assay

The CC_{50} of the compounds in treated cells was determined by measuring intracellular ATP levels. The sensitivity of this assay was confirmed by using saponin (Sigma-Aldrich) as a positive control, as its cytotoxicity was previously reported [47]. Specifically, 10^4 cells per well were seeded in 96-well flat bottom plates in total volume of 100 µL complete DMEM. 24 h post-seeding, cells were incubated with the compounds for 72 h at 37°C (5% CO_2) and lysed for ATP measurement. Calculation of the compound concentration causing 50% cell death (CC_{50}) was performed using cells treated with DMSO as control sample. CC_{50} values were determined by nonlinear regression analysis after converting the drug concentrations into log-X using Prism 5.0 software (GraphPad Software Inc.).

Measurement of intracellular ATP levels

ATP was measured using the ViaLight HS BioAssay kit (Lonza) according to the manufacturer's protocol in a GloMax 20/20 single-tube luminometer (Promega) for 1 s. ATP levels were normalized to total protein amounts.

Indirect immunofluorescence

Indirect immunofluorescence analysis of Con1 NS5A was performed as described elsewhere [46]. DNA was stained with propidium iodide (Sigma-Aldrich). Images were acquired with the Leica TCS-SP5II Two-photon Confocal Microscope with Spectra Physics Mai Tai infrared laser source.

Gel electrophoresis and Western blot analysis

Denaturing SDS-polyacrylamide gelelectrophoresis and Western blotting was performed as described elsewhere Dilutions of 1:1,000 for HCV NS5A monoclonal antibody 9E10 (kindly provided by C.M. Rice, The Rockefeller University, NY) [48], and 1:6000 for pan-actin mouse monoclonal antibody (Chemicon International) were used.

Total RNA extraction and quantification of viral replicons

Total RNA was extracted from Huh5-2 cells using TRIzol reagent (Ambion), according to the manufacturer's instructions. Replicon RNA was quantified with reverse-transcription (RT) and quantitative real-time polymerase chain reaction (qPCR). For RT, the Con1 IRES specific primer 5'-GGATTCGTGCTCATGGTGCA-3' (reverse) and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega) were used. For qPCR, the Con1 IRES specific primers 5'-GGCCTTGTGGTACTGCCTGATA-3' (forward) and 5'-GGATTCGTGCTCATGGTGCA-3' (reverse) and KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems) were used. The housekeeping gene YWHAZ was employed as an internal control (primers 5'-GCTGGTGATGACAAGAAAGG-3' and 5'-GGATGTGTTGGTTGCATTTCCT -3').

Development of Drug-Resistance Mutations

Huh5-2 replicon cells were treated with **15d** at 40-160 μM (concentrations 1-4 times of the EC_{50} value) in the presence of 500 μg/mL G418 for 16 passages. **15d** concentration was gradually increased (1-2-4x EC_{50}). Cells treated with DMSO were used as control. The resulting cell populations were used for anti-HCV assay to quantify **15d** activity, or lysed for RNA isolation.

HCV RNA amplification by long template RT-PCR

RNA isolation from cells treated with **15d** at 1-2-4xEC_{50} or DMSO, was performed using TRIzol reagent (Ambion), according to manufacture's instructions. Reverse transcription of HCV RNA from treated replicon cells was performed using MMLV reverse transcriptase (Promega). Specifically, 0.5 μg total RNA was mixed with 80 pmol reverse primer A9413 (5'-CAGGATGGCCTATTGGCCTGGAG-3') and transcription reaction solution (5x MMLV buffer,

2mM nucleotide mix (Invitrogen) and H_2O) in 22 µL total volume and incubated at 90°C for 3min. Next, 176 U MMLV and 21.6 U rRNasin (Promega) were added and reverse transcription was performed for 1h at 42°C.

Long template PCR was carried out using TaKaRa LA taq polymerase with CG buffers (TaKaRa). The Con1 HCV protein coding sequence (NS3-NS5B) was amplified in four segments of 1.5-2 kb with overlapping regions, using specific primers (Supplementary Table 1). 2 µL cDNA were added to 50µL reaction mixture (0.5 µM forward primer, 0.5 µM reverse primer, 1.6 mM nucleotide mix, 1x buffer 1, 2U polymerase, H_2O up to 50 µL) and polymerase chain reaction was performed following the instructions of the manufacturer.

Purification and Next Generation Sequencing (NGS) of PCR products encoding the NS3-NS5B region of Con1 replicon

The PCR products were separated via agarose gel-electrophoresis and products of appropriate size were purified with the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) according the instructions of the manufacturer. Samples were further purified with Agencourt AMPure XP beads (Beckman Coulter). Following purification, PCR products from each sample were quantified with a Qubit2.0 Fluorometer (Life Technologies) and Qubit dsDNA HS Assay kit (Life Technologies), and pooled together at equimolar ratios. The Ion Xpress™ Plus Fragment Library Kit (Life Technologies) was used to shear 100 ng of the pooled cDNA into ~200-bp fragments. The sheared cDNA fragments were purified with the Agencourt AMPure XP beads, ligated with Ion Xpress™ Barcode Adapters Kits (Life Technologies) to prepare the multiple cDNA libraries, size selected with E-Gel™ SizeSelect™ Agarose Gels (Life Technologies) and further purified with Agencourt AMPure XP beads. The quality and quantity of the libraries were assessed by using Qubit2.0 Fluorometer (Life Technologies) and the Ion Library TaqMan™ Quantitation Kit (Life Technologies).

The libraries were then enriched using the Ion OneTouch™ 2 System (Life Technologies) and loaded on an Ion 318™ Chip on the Ion PGM™ System (Life Technologies). The data

obtained were analyzed using the Ion PGM™ System software (Life Technologies) to identify resistant mutations.

Statistical analysis

In all diagrams, bars represent mean values of at least two independent experiments in triplicate or tetraplicate. Error bars represent standard deviation. Only results subjected to statistical analysis using Student's t-test with $p \leq 0.05$ were considered as statistically significant and presented. Statistical calculations were carried out using Excel Microsoft Office®.

Results and discussion

Chemistry

The target nucleosides were prepared from 1,6-diaminopyridine (**1**, Scheme 1) which was acetylated and then nitrated to give compound **3**. The nitropyridine **3** was selectively deacetylated and provided the 2-amino-acetamide **4**, in a 50% yield, together with traces of the fully deprotected 2,6-diamino-3-nitropyridine. The nitroderivative **4** was catalytically reduced with the use of palladium on carbon as catalyst. The resulting unstable diamine was not isolated, but upon treatment with glyoxal was converted to the pyrido[2,3-*b*]pyrazine **5**. Attempts to nitrate the pyridopyrazine **5** were not successful, since the nitroderivative **6** was obtained only with the use of a mixture of acetic anhydride and fuming nitric acid, albeit in very low yield (6%).

Consequently, we followed a modified synthetic procedure, using the pyridineacetamide **4**, which upon catalytic reduction and treatment of the resulting diaminopyridine with triethylorthoformate, was converted to the imidazopyridine **7**. Compound **7** was nitrated to result in the nitroderivative **8**, which was first catalytically reduced and the resulting diamine was ring-closed as mentioned above and converted to the tricyclic base **9**.

Compound **9** was introduced to a Vorbrüggen type glycosylation and reacted with 1,2,3,5-tetra-*O*-acetyl-β-D-ribofuranose in the presence of *N,O*-bis(trimethylsilyl)acetamide and trimethylsilyl trifluoromethanesulfonate (TMSOTf, Scheme 2) [49, 50]. Both regio-isomers

10 and **11** were isolated from the glycosylation reaction, each as a mixture of the corresponding α/β anomeric forms. Upon repeated chromatographic purifications, we managed to isolate pure the predominant β-anomers of each regio-isomer, since the percentage of α-anomers in each mixture was very low. The unambiguous characterization of the nucleosides **10** and **11** was effected through 1D and 2D-NMR experiments. More precisely, concerning compound **11**, we observed clear correlation peaks between the aromatic H-9 with protons of the ribosyl moiety, in the corresponding nOe spectral data, indicating the position of ribosylation. On the other hand, the configuration at C-1' was assigned on the basis of nOe experiments and concerning both **10** and **11** we recorded strong couplings between the anomeric protons of each compound with the corresponding H-4'.

The acetyl protecting groups were easily removed upon treatment of **10** and **11** with methanolic ammonia, to afford quantitatively the deprotected derivatives **12** and **13** respectively. The tricyclic nucleoside **10** was then subjected to electrophilic substitution, using *N*-chlorosuccinimide and was converted to the 9-chloroderivative **14** in good yield (49%). The site of halogenation was confirmed using nOe spectral data, where clear correlation peaks were observed between the ribosyl H-1', H-2', H-3' and the aromatic H-2. Compound **14** was used for the synthesis of the target aminoderivatives, using suitable primary amines bearing linear or branched aliphatic or aromatic groups with the aim to extract structure-activity relationships. The chloride **14** was thus coupled with the selected amines, in the presence of 2-dicyclohexylphosphino-2',4',6'-triisopropylbiphenyl (X-phos) and tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$), followed by the concomitant deprotection of the acetyl groups in alkaline media, to provide the target nucleosides **15a-e** in reasonable yields.

Biological evaluation

Screening of nucleoside analogues in HCV replicon cells

The effects of the synthesized nucleoside analogues on HCV RNA replication and cell viability were determined in the Huh5-2 stable cell line containing the firefly luciferase-encoding subgenomic replicon of the HCV genotype 1b Con1 strain (Figure 2A). The initial evaluation of all compounds was performed at a concentration of 100 μM by measuring luciferase activity. As shown in Figure 2B, compounds **12** and **13** that do not possess a 9-alkyl/arylamino substitution, were inactive. However, the 9-aminoderivatives **15a-e** inhibited HCV replication with different potency. The furylmethyl- derivative **15e** was the least active compound. Among the alkylamino- substituted analogues, the branched isopentylamino-derivative **15b** appeared to be the most active, followed by the corresponding isopentenyl derivative **15c**, while the n-butyl derivative **15a** possessed the lowest potency. The most promising analogue identified was the benzylamino- derivative **15d**, which was thus selected for further evaluation. This compound reduced replication of genotype 1b HCV with an EC_{50} of 40.22 μM (Figure 2C, Table 1), and showed promising safety since the half maximal cytotoxic concentration (CC_{50}) was higher than 200 μM (the highest concentration tested) (Figure 2D). Thus, even if its activity can be considered moderate, it exhibited a selectivity index (SI: CC_{50}/EC_{50}) higher than 4.97. Compound **15d** was also evaluated against subgenomic replicons of genotypes 2a (strain JFH1), 3a (strain S52), and 4a (strain ED43), showing antiviral activity only against genotype 3a with an EC_{50} value of 73.56 μM (Table 1). This activity against 3a is of importance as patients with genotype 3 infection still are most difficult to treat especially in patients with liver cirrhosis [51].

The inhibition profiles measured by the luciferase assay in genotype 1b were confirmed using western blot (Figure 3A) and indirect immunofluorescence against NS5A (Figure 3B). Consistently, replicon RNA levels were significantly reduced in the presence of **15d**, with an EC_{50} value of 25 μM (Figure 3C).

Validation of 15d against full-length HCV viruses

The effect of **15d** on HCV replication was confirmed in the context of full length HCV. *In vitro* transcripts of reporter viruses of the HCV isolates H77S.3 (genotype 1a) or HCV-N.2 (genotype 1b) (Figure 4A) were electroporated into Huh7-Lunet cells. Of note, for the genotype 1b virus an EC_{50} value of 52.72 µM was calculated (Figure 4B), similar to the one measured for the subgenomic replicon of the Con1 isolate (Table 1), confirming the antiviral activity of this compound. A higher EC_{50} value (83.62 µM) was observed for the genotype 1a virus. No cytotoxicity was detected even at the highest compound concentrations tested (Figure 4C).

Determining the viral target of 15d by resistance mutation analysis

To investigate the possible mechanism of action for **15d** on HCV replication, we selected for drug resistance by using Huh5-2 replicon cells that were cultured for 16 passages in the presence of G418 to maintain the replicon and **15d** at increasing concentrations up to 4-fold of EC_{50} (1-2-4xEC_{50}, Figure 5A). While direct treatment of cells with 4 times the EC_{50} of **15d** resulted in cell death after 3 passages, gradual increase of **15d** concentration, allowed the selection of drug-resistant colonies after 8 cell passages up to 4xEC_{50}. In comparison to cells treated for the same number of passages with G418 and DMSO only, we observed an about 1.7-fold increase of the EC_{50} value for **15d** in compound-treated cells, as determined by measuring luciferase activity (Figure 5B). This suggests the development of a slight viral resistance to the inhibitor, which prompted us to determine the coding sequence of NS3 to NS5B from the replicon system by using next generation sequencing (NGS). 7 non-synonymous amino acid substitutions (E295G, V298D, I352T, P353S, S414P, D443G and C446R) were detected after the 16th passage with **15d**, all in positions located in the C-terminal part of NS5A (Figure 5D). Direct comparison of the replication fitness of the HCV replicon in the **15d** and DMSO-adapted cell populations showed similar replication levels (Figure 5C). This suggests that the selected mutations do not increase the fitness of the virus.

These results indicate a possible resistance via NS5A and imply that these nucleoside analogues act through an NS5B-independent mechanism. Of the mutations detected in NS5A, V298D, I352T, P353S and S414P were present only in **15d**-treated cells, whereas E295G, D443G and C446R were present also in DMSO-treated cells, but at a much lower frequency, as compared to the inhibitor-treated cells. These mutations are not among those reported in the literature as cell-culture adaptive ones [52-56]. Mutations E295G and V298D are positioned in domain II (D2), I352T and P353S in low-complexity sequence II (LCSII) and S414P, D443G and C446R in D3. Thus, **15d**-resistance mutations appear in different domains than D1 which is previously reported to be targeted by the NS5A-specific DAAs. As D2 and D3 have been implicated in a broad spectrum of interactions with cellular and other viral proteins [22], our results imply that a cellular target of **15d** is also possible. This possibility is also favored by the low increase (1.7-fold) in the EC_{50} value of **15d** observed after the drug-resistance selection, as the high barrier of resistance is characteristic of host-targeting inhibitors [57]. However, we cannot exclude the possibility that **15d** targets NS5A-NS5B interactions, even by targeting NS5B, as mutations E295G and V298D are within a region essential for the binding of NS5B [58, 59]. In addition, E295G and V298D are at the carboxy-terminal part of a region implicated in the interaction of NS5A with protein kinase R (PKR), an IFN-stimulated gene, and thus might inhibit PKR activation [60]. Interestingly, I352T and P353S create two possible phosphorylation positions in LCSII, with threonine at position 352 being a native phosphorylation site in the JFH1 strain (genotype 2a) with a as yet undefined role [61]. The presence of T352 in JFH1 might be a reason for the difference in the activity of **15d** in this strain as compared to Con1. On the other hand, S414P removes a putative phosphorylation site that is involved in basal phosphorylation and has been implicated in virus assembly [62]. All these imply a possible shift in NS5A phosphorylation, under the pressure of compound **15d**. Furthermore, I352T and P353S are contained in a conserved polyproline motif (PxxPxR) which is necessary for NS5A interaction with Src Homology 3 domain-containing proteins (Grb2 GTPase, Lyn and Fyn kinases). This interaction modulates host-cell signalling pathways, playing subtle roles in HCV replication

[29, 63]. However, these adaptive mutations are not on residues important for the motif function. Interestingly, mutation S414P in D3 has been previously associated with viral resistance to interferon through impairing STAT1 phosphorylation, although it is not positioned within the interferon sensitivity determining region [64]. In the same study, the **15d**-adaptive mutation E295G has also been developed in one of the IFN-resistant clones, although no further correlation with IFN resistance was made. In general, it is known that D3 region directly binds and inhibits STAT1, suppressing type I IFN signaling [65]. The same aminoacid position S414 has also been shown with biochemical assays to participate in the interaction of NS5A with the vesicle-associated membrane protein-associated protein B, which is important for the anchoring of the HCV RNA replication machinery onto the ER [66]. Moreover, positions S414 and D443 have been implicated in the binding of Cyclophilin A, a peptidylprolyl isomerase essential for HCV replication, to NS5A D3, based on chemical shift perturbation assays [28]. Finally, the aminoacid sequence directly upstream of D443 varies between the genotypes tested, with JFH1 and ED43 lacking one and two aminoacids respectively, as compared to Con1 and S52 (data not shown), which might be a possible reason for the lack of **15d** activity in genotypes 2a and 4a.

Conclusion

In conclusion, we have synthesized a series of new tricyclic nucleoside derivatives and have examined their inhibitory potential against HCV. The compounds were prepared from 1,6-diaminopyridine, which was converted first to 5-acetamidoimidazo[4,5-*b*]pyridine and then to 3*H*-imidazo[4',5':5,6]pyrido[2,3-*b*]pyrazine. The former analogue provided two regio-ribosides and through halogenation of the suitable isomer we obtained a number of 9-aminosubstituted nucleosides as well. We have identified the 9-benzylamino-substituted derivative **15d**, reducing HCV replication with a selectivity index higher than 4.97 (in 1b genotype). Based on resistance mutation studies we suggest that this interesting lead compound most probably possesses a NS5A-dependent mechanism of action, through a

different region than the one targeted by DAAs, which is unprecedented for nucleoside derivatives.

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Conflict of interest

The authors declare no conflict of interest.

References

1. Hoofnagle J.H. (2002) Course and outcome of hepatitis C. Hepatology;36:S21-29.

2. Wilder J., Patel K. (2014) A Review of the Natural History of Chronic Hepatitis C Infection. N Am J Med Sci;7: 1-7.

3. World Health Organization, 2016, Hepatitis C http://www.who.int/mediacentre/factsheets/fs164/en/**.**

4. Chauhan V., Goyal K., Singh M.P. (2016) HCV Vaccine: How Far are We? J Hum Virol & Retrovirol;3:00084.

5. Soriano V., Peters M.G., Zeuzem S. (2009) New therapies for hepatitis C virus infection. Clin Infect Dis;48:313-320.

6. Scheel T.K., Rice C.M. (2013) Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. Nat Med;19:837-849.

7. Wakita T., Pietschmann T., Kato T., Date T., Miyamoto M., Zhao Z., Mutrhy K., Habermann A., Krausslich H.G., Mizokami M., Bartenschlager R., Liang T.J. (2005) Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med;11:791-796.

8. Lohmann V., Korner F., Koch J., Herian U., Theilmann L., Bartenschlager R. (1999) Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science;285:110-113.

9. http://hepatitiscnewdrugresearch.com/approved-treatments-for-hepatitis-c.html**.**

10. European Association for Study of the Liver: http://www.easl.eu/research/our-contributions/clinical-practice-guidelines/detail/recommendations-on-treatment-of-hepatitis-c-2015

11. The American Association for the Study of Liver Disease: http://www.hcvguidelines.org**.**

12. Barth H. (2015) Hepatitis C virus: Is it time to say goodbye yet? Perspectives and challenges for the next decade. World J Hepatol;7:725-737.

13. King A.M.Q. (2012) Family - Flaviviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, editors *Family - Flaviviridae*. San Diego: Elsevier: p. 1003-20.

14. Simmonds P., Bukh J., Combet C., Deléage G., Enomoto N., Feinstone S., et al. (2015) Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology;42:962-973.

15. Bartenschlager R., Lohmann V., Penin F. (2013) The molecular and structural basis of advanced antiviral therapy for hepatitis C virus infection. Nat Rev Microbiol;11:482-496.

16. Moradpour D., Penin F., Rice C.M. (2007) Replication of hepatitis C virus. Nat Rev Microbiol;5:453-463.
17. Scola P.M., Sun L.Q., Wang A.X., Chen J., Sin N., Venables B.L., et al. (2014) The discovery of asunaprevir (BMS-650032), an orally efficacious NS3 protease inhibitor for the treatment of hepatitis C virus infection. J Med Chem;57:1730-1752.
18. Summa V., Ludmerer S.W., McCauley J.A., Fandozzi C., Burlein C., Claudio G., et al. (2012) MK-5172, a selective inhibitor of hepatitis C virus NS3/4a protease with broad activity across genotypes and resistant variants. Antimicrob Agents Chemother;56:4161-4167.
19. Lesburg C.A., Cable M.B., Ferrari E., Hong Z., Mannarino A.F., Weber P.C. (1999) Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat Struct Biol;6:937-943.
20. Zhang J., Nguyen D., Hu K.Q. (2016) Chronic Hepatitis C Virus Infection: A Review of Current Direct-Acting Antiviral Treatment Strategies. N Am J Med Sci;9:47-54.
21. Issur M., Gotte M. (2014) Resistance patterns associated with HCV NS5A inhibitors provide limited insight into drug binding. Viruses;6:4227-4241.
22. Ross-Thriepland D., Harris M. (2015) Hepatitis C virus NS5A: enigmatic but still promiscuous 10 years on! J Gen Virol;96:727-738.
23. Evans M.J., Rice C.M., Goff S.P. (2004) Phosphorylation of hepatitis C virus nonstructural protein 5A modulates its protein interactions and viral RNA replication. Proc Natl Acad Sci U S A.;101.13038-13043.
24. Moradpour D., Penin F. (2013) Hepatitis C virus proteins: from structure to function. Curr Top Microbiol Immunol;369:113-142.
25. Tellinghuisen T.L., Marcotrigiano J., Rice C.M. (2005) Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature;435:374-379.
26. Reiss S., Harak C., Romero-Brey I., Radujkovic D., Klein R., Ruggieri A., Rebhan I., Bartenschlager R., Lohmann V. (2013) The lipid kinase phosphatidylinositol-4 kinase III alpha regulates the phosphorylation status of hepatitis C virus NS5A. PLoS Pathog;9:e1003359.
27. Hanoulle X., Badillo A., Wieruszeski J.M., Verdegem D., Landrieu I., Bartenschlager R., Penin F., Lippens G. (2009) Hepatitis C virus NS5A protein is a substrate for the peptidyl-prolyl cis/trans isomerase activity of cyclophilins A and B. J Biol Chem;284:13589-13601.
28. Verdegem D., Badillo A., Wieruszeski J.M., Landrieu I., Leroy A., Bartenschlager R., Penin F., Lippens G., Hanoulle X. (2011) Domain 3 of NS5A protein from the hepatitis C virus has intrinsic alpha-helical propensity and is a substrate of cyclophilin A. J Biol Chem;286:20441-20454.
29. Macdonald A., Crowder K., Street A., McCormick C., Harris M. (2004) The hepatitis C virus NS5A protein binds to members of the Src family of tyrosine kinases and regulates kinase activity. J Gen Virol;85:721-729.
30. Amako Y., Igloi Z., Mankouri J., Kazlauskas A., Saksela K., Dallas M., Peers C., Harris M. (2013) Hepatitis C virus NS5A inhibits mixed lineage kinase 3 to block apoptosis. J Biol Chem;288:24753-24763.
31. Belema M., Meanwell N.A. (2014) Discovery of daclatasvir, a pan-genotypic hepatitis C virus NS5A replication complex inhibitor with potent clinical effect. J Med Chem;57:5057-5071.
32. Tite T., Lougiakis N., Skaltsounis A.L., Marakos P., Pouli N., Tenta R., Balzarini J. (2009) The Application of Mitsunobu Cyclization for the Synthesis of 2 ',3 '-Dideoxy-C-Nucleosides Designed as Didanosine Analogues. Synlett;1741-1744.
33. Tite T., Lougiakis N., Myrianthopoulos V., Marakos P., Mikros E., Pouli N., Tenta R., Fragopoulou E., Nomikos T. (2010) Design and synthesis of new C-nucleosides as potential adenosine deaminase inhibitors. Tetrahedron;66:9620-9628.
34. Lougiakis N., Marakos P., Pouli N., Fragopoulou E., Tenta R. (2015) Synthesis of new nebularine analogues and their inhibitory activity against adenosine deaminase. Chem Pharm Bull;63:134-142.

35. Leonard N.J., Sprecker M.A., Morrice A.G. (1976) Defined dimensional changes in enzyme substrates and cofactors. Synthesis of lin-benzoadenosine and enzymatic evaluation of derivatives of the benzopurines. J Amer Chem Soc;98:3987-3994.

36. Amblard F., Fromentin E., Detorio M., Obikhod A., Rapp K.L., McBrayer T.R., Whitaker T., Coats S.J., Schinazi R.F. (2009) Synthesis, antiviral activity, and stability of nucleoside analogs containing tricyclic bases. Eur J Med Chem;44:3845–3851.

37. Wauchope O.R., Tomney M.J., Pepper J.L., Korba B.E., Seley-Radtke K.L. (2010) Tricyclic 2′-C-Modified Nucleosides as Potential Anti- HCV Therapeutics. Org Lett;12:4466–4469.

38. Friebe P., Boudet J., Simorre J.P., Bartenschlager R. (2005) Kissing-loop interaction in the 3' end of the hepatitis C virus genome essential for RNA replication. J virol;79:380-392.

39. Blight K.J., McKeating J.A., Rice C.M. (2002) Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J virol;76:13001-13014.

40. Vrolijk J.M., Kaul A., Hansen B.E., Lohmann V., Haagmans B.L., Schalm S.W., Bartenschlager R. (2003) A replicon-based bioassay for the measurement of interferons in patients with chronic hepatitis C. J Virol Methods;110:201-209.

41. Jo J., Aichele U., Kersting N., Klein R., Aichele P., Bisse E., Sewell A.K., Blum H.E., Bartenschlager R., Lohmann V., Thimme R. (2009) Analysis of CD8+ T-cell-mediated inhibition of hepatitis C virus replication using a novel immunological model. Gastroenterology;136:1391-1401.

42. Saeed M., Scheel T.K., Gottwein J.M., Marukian S., Dustin L.B., Bukh J., Rice C.M. (2012) Efficient replication of genotype 3a and 4a hepatitis C virus replicons in human hepatoma cells. Antimicrob Agents Chemother;56:5365-5373.

43. Shimakami T., Welsch C., Yamane D., McGivern D.R., Yi M., Zeuzem S., Lemon S.M. (2011) Protease inhibitor-resistant hepatitis C virus mutants with reduced fitness from impaired production of infectious virus. Gastroenterology;140:667-675.

44. Shimakami T., Honda M., Shirasaki T., Takabatake R., Liu F., Murai K., Shiomoto T., Funaki M., Yamane D., Murakami S., Lemon S.M., Kaneko S. (2014) The acyclic retinoid Peretinoin inhibits hepatitis C virus replication and infectious virus release in vitro. Sci Rep;4:4688.

45. Kaul A., Woerz I., Meuleman P., Leroux-Roels G., Bartenschlager R. (2007) Cell culture adaptation of hepatitis C virus and in vivo viability of an adapted variant. J virol;81:13168-13179.

46. Vassilaki N., Friebe P., Meuleman P., Kallis S., Kaul A., Paranhos-Baccala G., Leroux-Roels G., Mavromara P., Bartenschlager R. (2008) Role of the hepatitis C virus core+1 open reading frame and core cis-acting RNA elements in viral RNA translation and replication. J virol;82:11503-11515.

47. Lee J., Lim S., Kang S.M., Min S., Son K., Lee H.S., Park E.M., Ngo H.T.T., Tran H.T.L., Lim Y-S., Hwang S.B. (2012) Saponin inhibits hepatitis C virus propagation by up-regulating suppressor of cytokine signaling 2. PloS ONE;7: e39366.

48. Lindenbach B.D., Evans M.J., Syder A.J., Wolk B., Tellinghuisen T.L., Liu C.C., Maruyama T., Hynes R.O., Burton D.R., McKeating J.A., Rice C.M. (2005) Complete replication of hepatitis C virus in cell culture. Science;309:623-626.

49. Vorbrüggen H., Krolikiewicz K., Bennua, B. (1981) Nucleoside Synthesis with Trimethylsilyl Triflate and Perchlorate as Catalysts. Chem Ber;114:1234-1255.

50. Vorbrüggen H., Bennua B. (1978) New simplified nucleoside synthesis. Tetrahedron Lett;19:1339-1342.

51. Gondeau C., Pageaux G.P., Larrey D. (2015) Hepatitis C virus infection: Are there still specific problems with genotype 3? World J Gastroenterol;21:12101-12113.

52. Blight K.J., Kolykhalov A.A., Rice C.M. (2000) Efficient initiation of HCV RNA replication in cell culture. Science;290:1972-1974.

53. Krieger N., Lohmann V., Bartenschlager R. (2001) Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J virol;75:4614-4624.

54. Blight K.J., McKeating J.A., Marcotrigiano J., Rice C.M. (2003) Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J virol;77:3181-3190.

55. Lohmann V., Korner F., Dobierzewska A., Bartenschlager R. (2001) Mutations in hepatitis C virus RNAs conferring cell culture adaptation. J virol;75:1437-1449.

56. Lohmann V., Hoffmann S., Herian U., Penin F., Bartenschlager R. (2003) Viral and cellular determinants of hepatitis C virus RNA replication in cell culture. J virol;77:3007-3019.

57. Pawlotsky J.M. (2014) What are the pros and cons of the use of host-targeted agents against hepatitis C? Antiviral Res;105:22-25.

58. Ngure M., Issur M., Shkriabai N., Liu H.W., Cosa G., Kvaratskhelia M., Götte M. (2016) Interactions of the Disordered Domain II of Hepatitis C Virus NS5A with Cyclophilin A, NS5B, and Viral RNA Show Extensive Overlap. ACS infectious diseases;2:839-851.

59. Shirota Y., Luo H., Qin W., Kaneko S., Yamashita T., Kobayashi K., Murakami S. (2002) Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. J Biol Chem;277:11149-11155.

60. Gale M., Blakely C.M., Kwieciszewski B., Tan S.L., Dossett M., Tang N.M., Korth M.J., Polyak S.J., Gretch D.R., Katze M.G. (1998) Control of PKR protein kinase by hepatitis C virus nonstructural 5A protein: Molecular mechanisms of kinase regulation. Mol Cell Biol;18:5208-5218.

61. Ross-Thriepland D., Harris M. (2014) Insights into the complexity and functionality of hepatitis C virus NS5A phosphorylation. J virol;88:1421-1432.

62. Masaki T., Suzuki R., Murakami K., Aizaki H., Ishii K., Murayama A., Date T., Matsuura Y., Miyamura T., Wakita T., Suzuki T. (2008) Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. J virol;82:7964-7976.

63. Macdonald A., Mazaleyrat S., McCormick C., Street A., Burgoyne N.J., Jackson R.M., Cazeaux V., Shelton H., Saksela K., Harris M. (2005) Further studies on hepatitis C virus NS5A-SH3 domain interactions: identification of residues critical for binding and implications for viral RNA replication and modulation of cell signalling. J Gen Virol;86:1035-1044.

64. Noguchi T., Otsubaki T., Ando I., Ogura N., Ikeda S., Shimotohno K. (2008) Isolation and gene analysis of interferon alpha-resistant cell clones of the hepatitis C virus subgenome. Virology;375:424-432.

65. Kumthip K., Chusri P., Jilg N., Zhao L., Fusco D.N., Zhao H., et al. (2012) Hepatitis C virus NS5A disrupts STAT1 phosphorylation and suppresses type I interferon signaling. J virol;86:8581-8591.

66. Gupta G., Qin H., Song J. (2012) Intrinsically unstructured domain 3 of hepatitis C Virus NS5A forms a "fuzzy complex" with VAPB-MSP domain which carries ALS-causing mutations. PLoS One;7:e39261.

Figure Legends and Tables

Figure 1. Structures of Sofosbuvir and daclatasvir.

Scheme 1. Reagents and conditions: a) Ac_2O, CH_2Cl_2, r.t., 97%; b) HNO_3, H_2SO_4, 0 ˚C then r.t., 89%; c) HCl, MeOH, 0 ˚C then r.t., 50%; d) 1) Pd/C, H_2, EtOH, 50 psi, r.t., 2) glyoxal, EtOH, reflux, 96% for **5**, 56% for **9** (two steps); e) $HNO_{3(f.)}$, Ac_2O, -20 ˚C, 6%; f) 1) Pd/C, H_2, EtOH, 50 psi, r.t., 2) triethylorthoformate, HCl, r.t., 93% (two steps); g) HNO_3, H_2SO_4, 0 ˚C then 50 ˚C, 68%.

Scheme 2. Reagents and conditions: a) 1) *N,O*-bis(trimethylsilyl)acetamide, ACN, reflux, 2) tetra-*O*-acetyl-*β*-D-ribofuranose, TMSOTf, reflux, 31% for **10**, 7% for **11** (two steps); b) NH_3, CH_3OH, r.t., 82-87%; c) NCS, THF, r.t., 60%; d) 1) X-Phos, $Pd_2(dba)_3$, amine, dioxane, 90 ˚C, 2) NH_3, CH_3OH, r.t., 39-60% (two steps).

Figure 2. A) Schematic representation of the HCV genotype 1b bicistronic replicon stably expressed in Huh5-2 cells. **B)** Evaluation of nucleoside analogues **12,13** and **15a-e** against HCV genotype 1b RNA replication, as determined in Huh5-2 subgenomic replicon cells, seeded at 30% confluence and treated for 72 h with the compounds at a concentration of 100 µM. As a marker of viral RNA replication and cell viability, firefly luciferase (FLUC) activity and intracellular ATP levels respectively were determined and expressed as relative light units (RLU) per µg of total protein. Values from the compound-treated cells were expressed as a percentage of that obtained from cells that received the solvent DMSO (M). Bars represent mean values obtained from two separate experiments in triplicate. Error bars represent standard deviation (SD). **C,D)** Anti-HCV activity **(C)** and cytotoxicity **(D)** of analogue **15d** using dose–response curve analysis of 2-fold serial dilutions. Assays for FLUC activity and intracellular ATP levels, respectively, were performed in Huh5-2 replicon cells. Values from the compound-treated cells were expressed as a percentage of that obtained from cells that received the solvent DMSO (M). Saponin was used as a positive control in the cytotoxicity assay.

Figure 3. Inhibitory effect of compound **15d** on HCV protein expression and RNA replication in subgenomic replicon assays. **A)** Western blot for NS5A in Huh5-2 replicon cells treated with **15d** at 100, 50 and 25 µM, or DMSO (M). β-αctin was used as loading control and for normalization. **B)** Indirect immunofluorescence for NS5A in Huh5-2 replicon cells, treated with **15d** at 100 and 50 µM, or DMSO (M). Nuclei were stained with propidium iodide (PI; left column). Bar, 100 µm. **C)** Quantification of HCV RNA by RT-qPCR. Huh5-2 replicon cells were treated with **15d** at 100, 50 and 25 µM, or DMSO (M). Values from compound-treated cells are expressed as percentage of those obtained from cells that received the solvent DMSO (M). mRNA levels of the housekeeping gene YWHAZ were used for normalization.

Figure 4. Evaluation of **15d** against full-length HCV reporter viruses **A)** Schematic representation of the viral RNA constructs encoding Gaussia luciferase (GLuc) used. Effect of 15d on **B)** virus replication and **C)** cell viability of Huh7-Lunet cells electroporated with *in vitro* transcribed full length reporter viral RNA of genotype 1a (H77S.3) or 1b (HCV-N.2) and then treated for 72 h with **15d** at 2-fold serial dilutions, or DMSO. Gaussia luciferase activity (marker of viral RNA replication) from each viral sequence was quantified in the supernatant and normalized to total protein. Intracellular ATP levels (marker of cytotoxicity) were quantified in lysed cells and normalized to total protein. For each virus, values from compound-treated cells are expressed as percentage of DMSO-treated cells (M). Calculated EC_{50} values are given in the graph above their respective curve. Points represent mean values obtained from two separate experiments in tetraplicate. Error bars represent standard deviation (SD).

Figure 5. A) Selection for resistance against **15d**. Huh5-2 replicon cells were treated for 16 passages with **15d** at increasing concentrations up to 4-fold of the EC_{50}, or with DMSO only. The resulting cell populations were analyzed by measuring F-Luc activity (HCV RNA replication marker), or lysed for RNA isolation, RT-PCR and analysis of resistance mutations. **B)** Left panel: Reduced activity of **15d** after cell passaging. Replicon cells treated for 16 passages (16 p. treat.) with the inhibitor (1x-2x-4xEC_{50}) or DMSO were subsequently treated with **15d** at 100 and 50 µM or DMSO (M) for 72 h. F-Luc activity was quantified and normalized to total protein. For each population, values from the compound-treated cells are expressed as percentage of those obtained with DMSO-only treated cells (M). Right panel: IC_{50} values of **15d** after treatment for 16 passages with the inhibitor (1x-2x-4xEC_{50}) or DMSO. **C)** Direct comparison of the replication fitness between the **15d**-resistant and wild-type (DMSO) replicon cell populations after treatment for 16 passages with the inhibitor (1x-2x-4xIC50) or DMSO respectively. The values from DMSO cells were set to 1. **D)** Schematic representation of the hepatitis C virus NS5A protein, depicting the amino acid substitutions

identified in Con1 subgenomic replicons after 16 cell passages in the presence of **15d**. Mutations are shown in bold and their position is given in parenthesis. Sequences refer to the genotype 1b Con1 isolate (GenBank accession no. AJ238799). NS5A has been proposed to consist of three domains (labeled domains I, II, and III) with domains separated by low-complexity sequences (labeled LCS I and II). The position of the amino-terminal amphipathic helix serving as membrane anchor is shown (labeled helix).

Table 1. EC_{50}, CC_{50} and SI values of **15d** against genotypes 1b (Con1), 2a (JFH1), 3a (S52) and 4a (ED43) replicon cells.

Genotype/strain	EC_{50} (μM)	CC_{50} (μM)	SI
1b (Con1)	40.22	>200	>4.97
2a (JFH1)	>100	>200	-
3a (S52)	73.56	>200	>2.71
4a (ED43)	>100	>200	-