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3′-Deoxy Phosphoramidate Dinucleosides as Improved Inhibitors of Hepatitis C Virus Subgenomic Replicon and NS5B Polymerase Activity

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Phosphoramidate dinucleosides named "GC 3′-OH" series, carrying various phosphoramidate linkages have been previously reported as hepatitis C virus (HCV) inhibitors. To enhance the efficacy of these dinucleotides, we synthesized a novel "GC 3′-H" series as potential chain terminators. We showed that their inhibition potency is strongly increased by the introduction of novel neutral and bis-negatively charged phosphoramidate side chains. Their inhibitory effect on HCV NS5B polymerase was evaluated in vitro and in HCV subgenomic replicon containing Huh-6 cells. As expected, 3′-H compounds are more potent than their 3′-OH counterparts to inhibit HCV polymerase activity. The most potent inhibitor, a 5′-phosphorylated dinucleotide bearing a bis-negatively charged amino side chain (**7**), exhibits an IC_{50} value of 8 μM in vitro and EC_{50} value of 2.6 μM in the HCV subgenomic replicon system. A molecular structure model is presented to propose an interpretation of the gain afforded by the of 3′-H-cytidine modification.

Introduction

Hepatitis C is nowadays among the most severe viral diseases, with about 200 million of infected people worldwide.[1] The lack of a selective and efficient treatment for hepatitis C virus (HCV[a])-associated disease represents thus a global health problem. Since the discovery of the virus,[2,3] much effort has been made in characterizing the viral genome,[4] and the HCV viral life cycle,[5,6] in order to allow rational design of efficient antiviral therapies.[7] However, the sole currently approved treatment, combination therapy with PEGylated interferon-α and ribavirin, is only partly efficient[8] and associated with important side effects.[9] Despite the numerous obstacles, including the lack of robust cellular evaluation systems[10] and convenient animal models[11] as well as the emergence of resistance,[12] a number of antiviral agents, able to inhibit different steps of the HCV viral life cycle, have been developed during the past decade.[7,13−15] In our search for efficient and selective inhibitors of the HCV RNA-dependent-RNA-polymerase (RdRp) NS5B, we have recently described as HCV polymerase inhibitors several GC phosphoramidate dinucleosides named GC 3′-OH.[16] These compounds were rationally designed to enhance their inhibition properties by increasing their binding to active site of NS5B and to viral RNA: at least (a) GC is the complementary sequence of HCV 3′-end viral RNA negative strand, (b) a dinucleotide would act as a direct substrate to the enzyme without the need of any phosphorylation in cells, (c) a 2′-*O*-methyl guanosine was chosen to increase enzymatic and chemical stability of target compounds. These compounds exhibited inhibitory activity in an in vitro initiation assay on recombinant NS5B polymerase with IC_{50} value up to 25 μM and in cell culture assay on HCV subgenomic replicon with EC_{50} value up to 9 μM.

The structure−activity relationship (SAR) study highlighted that phosphoramidate dinucleosides bearing a 5′-phosphate (or -thiophosphate) group along with a neutral or a negatively charged side chain displayed the most potent inhibitory activity. In addition, we observed better potency for the Sp isomers compared to the Rp ones and to the diastereoisomeric mixtures. Then, these data allowed us to propose a model of the polymerase−phosphoramidate dinucleoside complex refined by molecular dynamic simulations, explaining the observed effects of the more potent phosphoramidate dinucleoside. In the present work and according to our previous model, we report the synthesis and the SAR study of a novel series of improved phosphoramidate dinucleosides named "GC 3′-H". The inhibitory potency of these new compounds was first enhanced thanks to

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[a] Abbreviations: HCV, hepatitis C virus; NS5B, non structural protein 5B; RdRp, RNA-dependent-RNA-polymerase; GC, guanosin-3′-yl-cytidin-5′-yl dinucleotide; SAR, structure−activity relationship; DMTr-Cl, 4,4′-dimethoxytrityl chloride; BSA, benzene sulfonyl acid; Boc, *tert*-butyloxy carbonyl; TFA, trifluoroacetic acid, Pal, palmitoyl; Cne, 2-cyanoethyl; GTP, guanosine triphosphate; CTP, cytidine triphosphate; Arg, arginine; Lys, lysine; NTP, nucleoside triphosphate; GMP, guanosine monophosphate; Asp, aspartate; Ser, serine; MD, molecular dynamics; pppGpC, 5′-triphosphate phosphodiester dinucleotide.

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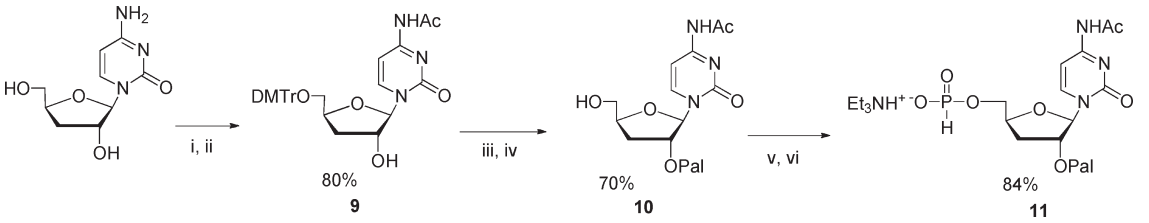
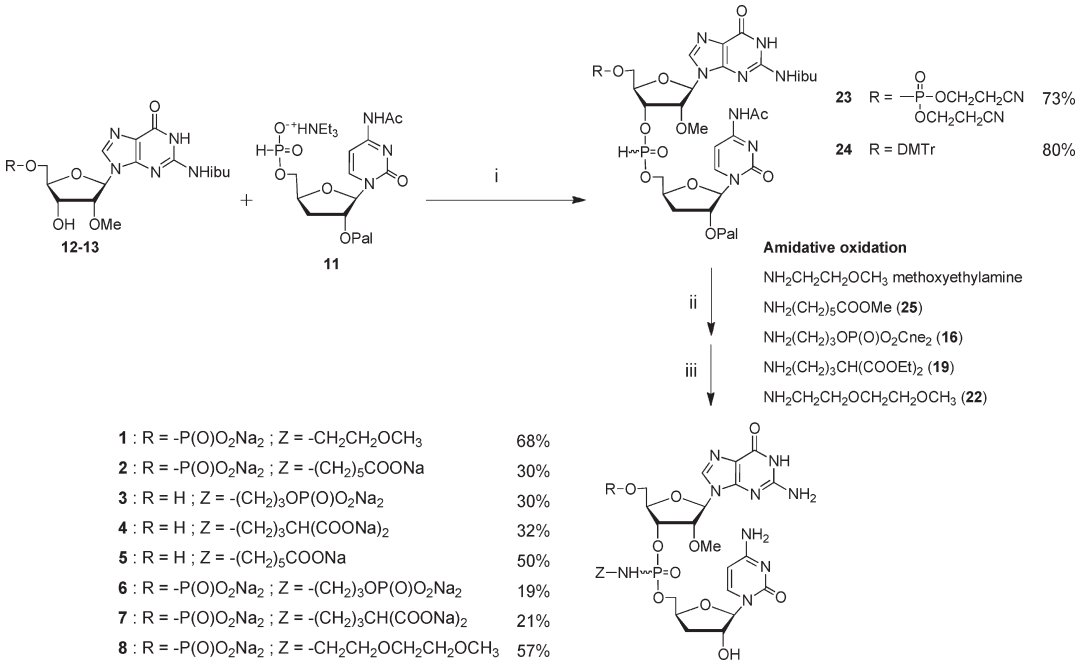
Scheme 1. Synthetic Pathway for the Synthesis of **11**[a]



[a] Reagents and conditions: (i) Ac_2O, DMF; (ii) DMTrCl, pyridine; (iii) PalCl, Pyridine; (iv) 2.5% benzenesulfonic acid in CH_2Cl_2/CH_3OH, 0 °C; (v) diphenylphosphite, pyridine; (vi) Et_3N, H_2O.

Scheme 2. Coupling of **12**−**13** and **11** Affording *H*-Phosphonate Diester Dimers **23**−**24**, Amidative Oxidations of **23**−**24**, and Deprotections Affording Target Compounds **1**−**8**[a]



1 : R = -P(O)O_2Na_2 ; Z = -$CH_2CH_2OCH_3$ 68%
2 : R = -P(O)O_2Na_2 ; Z = -$(CH_2)_5COONa$ 30%
3 : R = H ; Z = -$(CH_2)_3OP(O)O_2Na_2$ 30%
4 : R = H ; Z = -$(CH_2)_3CH(COONa)_2$ 32%
5 : R = H ; Z = -$(CH_2)_5COONa$ 50%
6 : R = -P(O)O_2Na_2 ; Z = -$(CH_2)_3OP(O)O_2Na_2$ 19%
7 : R = -P(O)O_2Na_2 ; Z = -$(CH_2)_3CH(COONa)_2$ 21%
8 : R = -P(O)O_2Na_2 ; Z = -$CH_2CH_2OCH_2CH_2OCH_3$ 57%

[a] Reagents and conditions: (i) Polystyrene-acyl chloride resin, DCM/pyridine −1:1 (v/v). (ii) Amine (methoxyethylamine, **25**, **16**, **19**, or **22**) CCl_4, pyridine. (iii) Deprotections: method A, 28% aqueous ammonia solution, 7 h at 50 °C; method B, MeOH and 80% acetic acid solution, 2 h at room temperature; method C, THF/acetonitrile −1:1 (v/v) and 0.4 M aqueous NaOH solution. (iv) RP-HPLC, then Dowex 50W X2 sodium form.

the introduction of a 3′-deoxycytidine moiety in order to exhibit a proper chain terminator effect.[17] In addition, on the basis of our previous SAR observations, we introduced some novel neutral and bis-negatively charged phosphoramidate side chains. These target compounds were then evaluated in an in vitro initiation assay on recombinant HCV polymerase NS5B and in Huh-6 cells containing an HCV subgenomic replicon. An extensive SAR was then established and the antiviral properties of the most potent compounds were investigated in detail. A molecular model of the NS5B polymerase active site−phosphoramidate dinucleoside complex was constructed to explain our results.

Results and Discussion

Chemistry. The phosphoramidate dinucleoside compounds (**1**−**8**) were prepared according to a synthetic strategy previously described by us,[16] involving coupling reaction between the 5′-*H*-phosphonate monoester of conveniently protected 3′-deoxycytidine **11** and previously described 2′-*O*-methylguanosine analogues **12**−**13**[18,19] (Scheme 2). First, **11** was prepared starting from 3′-deoxycytidine;[20] after introduction of an acetyl group at the N^4-position[21] and a palmitoyl group at the 2′ position, the *H*-phosphonate monoester function at the 5′ position was introduced after removal with benzene sulfonic acid (BSA) of the dimethoxytrityl (DMTr) group (Scheme 1).[22] The palmitoyl group was chosen to increase the lipophilicity allowing an easy extraction from water of **11**.

Coupling reaction between **11** and **12**−**13** was then performed, using solid-supported benzoyl chloride as a condensing reagent,[23] affording the corresponding dinucleoside *H*-phosphonate diesters **23**−**24**, which were further oxidized by CCl_4 in presence of various amines, providing, after appropriate deprotection and purification steps, target compounds (**1**−**8**) (Scheme 2). Amine **25**[16] was prepared according to previously reported procedure,[24] amines **16**, **19**,[25] and **22**[26] were prepared using a Boc-protecting strategy, followed by functionalization and deprotection by trifluoroacetic acid (TFA), and used as their TFA salts. Detailed synthetic procedures are given as Supporting Information.

For each target compound, a mixture of two diastereoisomers, exhibiting the Sp and the Rp absolute configurations on the asymmetric phosphorus atom of the phosphoramidate linkage, was obtained and this was confirmed by NMR and sometimes visible on the HPLC spectra (as two peaks). The mixture was directly tested for inhibitory evaluation. Henceforth, the more potent compounds tested as isomeric mixture

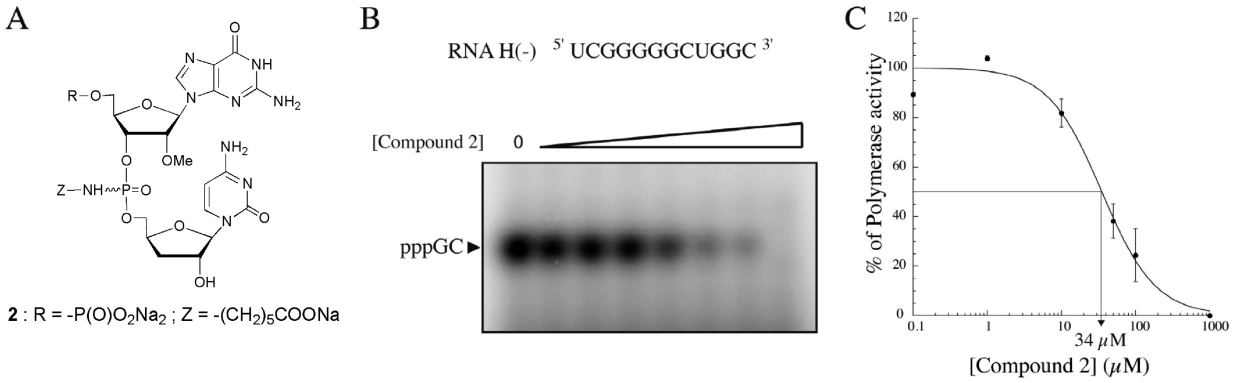


Figure 1. Introduction of a 3′-deoxycytidine moiety improved the inhibitory effect of GC phosphoramidate dinucleosides. (A) Structure of GC 3′-H compound. (B) Formation of initiation products by the HCV NS5B polymerase using template corresponding to the 3′ end of the HCV strand (−) genome in the presence of increasing concentration (0.1, 1, 10, 50, 100, 1000 μM) of compound **2**. Initiation products were separated by electrophoresis on denaturing sequencing gel. (C) pppGpC products obtained for various concentrations of compound **2** were quantified and values reported as a function of concentration. Curves corresponding to average values for at least three independent experiments are shown, and IC_{50} values calculated from these curves are indicated under the arrow.

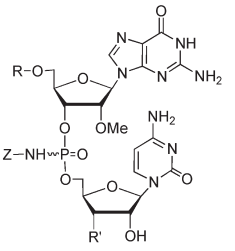
were further separated by HPLC on a semipreparative reverse phase C_{18} column, and each diastereoisomer was obtained as pure compound (see Supporting Information for HPLC and NMR examples). For all isolated diastereoisomers, a clear correlation was established between the retention times on RP-HPLC and the ^{31}P NMR chemical shift of the phosphoramidate bond, as previously noticed.[27] In analogy to the established behavior of dinucleoside phosphoramidate diastereoisomers,[28] the isomer with the shorter retention time on RP-HPLC (named fast) and with the more upfield ^{31}P chemical shift of the P−N bond (named "uf") was assigned as the diastereoisomer with the Rp configuration, while the other (named slow and "df") was assigned to the one with the Sp configuration. The validity of this assignment is supported by the published molecular model of a Sp isomer of a phosphoramidate dinucleoside docked within the active site of the HCV NS5B polymerase.[16]

In Vitro Evaluation of Dinucleotides Inhibiting De Novo Initiation of RNA Synthesis by HCV NS5B Polymerase. Effect of the Nature of 3′-Group of Cytidine Derivative on NS5B Polymerase Activity. To determine whether the synthesized GC 3′-H compounds (**1**−**8**) can inhibit initiation of RNA replication by HCV NS5B, a de novo initiation test was performed. This initiation step is defined as the formation of the first phosphodiester bond between two nucleotides, leading to a ribo-dinucleotide primer. The assay used as template a synthetic oligonucleotide corresponding to the 3′-end of the negative strand of the HCV genome. A mixture of GTP and [γ-^{32}P]-GTP was used as the initiating radiolabeled nucleotide. The formation of a GC dinucleotide 5′-triphosphate product (pppGpC) was monitored by inclusion of a CTP as elongating nucleotide in the reaction. Therefore, phosphoramidate dinucleosides **1**−**8**, bearing $^{5'}$-GC-$^{3'}$ nucleobases, were designed to mimic the formation of the first phosphodiester bond and thus to target the initiation step of the RNA replication by HCV NS5B. Hence, we measured the production of the pppGpC product and the ability of GC 3′-H compounds (**1**−**8**) to inhibit this reaction. The pppGpC product formation was quantified and allowed us to determine the 50% inhibitory concentration (IC_{50}) values of the different compounds (Figure 1 and Table 1). An example of such an experiment is presented in Figure 1 in which inhibitory effect of compound **2** is analyzed. This compound was structurally comparable to the previously reported 3′-OH equivalent derivatives compound **2′** that exhibited an IC_{50} value of 83 μM (Table 1). As expected, when a GC 3′-H compound is added to the reaction in increasing amounts, the accumulation of pppGpC product is decreased, reflecting the inhibition of RNA synthesis by HCV polymerase at the initiation step (Figure 1). As expected, all IC_{50} values for GC 3′-H compound, ranging from 8 to 67 μM, were shown to be lower than those previously obtained with equivalent GC 3′-OH compounds that ranged from 33 to >300 μM.[16]

Our previous SAR study[16] revealed that the highest inhibition potency of the GC 3′-OH was obtained when the dinucleoside phosphoramidates bear (i) a negatively charged or a neutral phosphoramidate side chain and (ii) a 5′-phosphate (or -thiophosphate) modification. To improve the inhibitory potency of our previous compounds, the 3′-H equivalent compounds were designed as proper chain terminators, synthesized, and their antiviral evaluation was performed. Hence, the present compounds **1** and **2** are structurally comparable to their previous 3′-OH equivalent derivatives **1′** and **2′** in regards to the 5′-terminal and the phosphoramidate side chain features. As shown in Figure 1 and Table 1, compounds **1** and **2** exhibited a significant inhibitory effect on de novo NS5B initiation. This result indicates that the newly designed GC 3′-H compounds were able to efficiently compete with the initiating nucleotide GTP for binding to the NS5B polymerase and were able to inhibit RNA synthesis by HCV NS5B at the initiation step, as previously reported for their 3′-OH analogues. Moreover, IC_{50} values of 29 and 34 μM of GC 3′-H compounds **1** and **2** showed an improved activity of 2−5-fold as compared with the GC 3′-OH parent compounds **1′** and **2′** (respectively 140 and 83 μM).[16] These results clearly show that removal of the 3′-OH group, and hence the possibility to elongate the modified dinucleoside, was able to increase the inhibition potency of GC phosphoramidate dinucleosides.

Effect of Side Chain on NS5B Polymerase Activity. To better understand the impact of the side chain functionality on the inhibition of HCV NS5B, and hence improve the potency of our new series, we designed and synthesized new derivatives bearing a larger neutral ethylene glycol ether

Table 1. Introduction of a 3′-Deoxycytidine Moiety Improved the Inhibitory Effect of GC Phosphoramidate Dinucleosides[a]

	Compd	Rp:Sp	Structure	IC_{50}(μM)
	**1'**	1:1	R = -P(O)O_2 Na_2 R' = OH Z = -$(CH_2)_2OCH_3$	140 ± 27
	2'	1:1	R = -P(O)O_2 Na_2 R' = OH Z = -$(CH_2)_5COO^-{}^+Na$	83 ± 13
	1	1:1	R = -P(O)O_2 Na_2 R' = H Z = -$(CH_2)_2OCH_3$	29 ± 11
	2	1:2.3	R = -P(O)O_2 Na_2 R' = H Z = -$(CH_2)_5COO^-{}^+Na$	34 ± 8
	3	1:1.5	R = H R' = H Z = -$(CH_2)_3OP(O)O_2^-Na_2^+$	21 ± 6
	4	1:1.5	R = H R' = H Z = -$(CH_2)_3CH(COO^-{}^+Na)_2$	19 ± 9
	5	1:2.7	R = H R' = H Z = -$(CH_2)_5COO^-{}^+Na$	67 ± 7
	6	1:1.3	R = -P(O)O_2Na_2 R' = H Z = -$(CH_2)_3OP(O)O_2^-Na_2^+$	10 ± 3
	7	1:2.3	R = -P(O)O_2Na_2 R' = H Z = -$(CH_2)_3CH(COO^-{}^+Na)_2$	8 ± 3
	8	1:1.4	R = -P(O)O_2 Na_2 R' = H Z = -$(CH_2)_2O(CH_2)_2OCH_3$	18 ± 4

[a] In vitro evaluation (IC_{50} values) of neutral (**1**, **1′**, and **8**) or negatively charged GC 3′-H and 3′-OH (**2**, **2′**, **3**, **4**, **5**, **6**, **7**, and **8**) and harboring a 5′-OH (**3**, **4**, and **5**) or a 5′-phosphate monoester group (**2**, **2′**, **6**, and **7**). All data represent average values for at least three separated experiments. The Rp:Sp column represents the ratio between each diastereoisomers in the mixture.

side chain (**8**) or negatively charged side chains bearing two charges (**6** and **7**). Confirming our expectations, compound **8** exhibited better inhibition than compound **1**, with IC_{50} values of 18 and 29 μM, respectively. Compounds **6** and **7**, bearing either a monophosphate or a dicarboxylic malonate amino side chain, exhibited the most potent inhibition so far, with IC_{50} values of 10 and 8 μM, respectively. According to the results described for GC 3′-OH compounds,[16] the presence of a neutral (methoxyethyl) or mono negatively charged (carboxypentyl) side chain is crucial to inhibition potency. In addition, we now demonstrate that about 3-fold gain of activity is observed when the amino side chain bears two negative charges (compounds **6** and **7**) instead of one (compound **1**). These data are consistent with our previous molecular model,[16] revealing that the only way to accommodate the phosphoramidate side chain in the HCV NS5B active site is to direct it toward the nucleotide entry channel paved with positively charged Arg/Lys residues. Thus, increasing the acidity of the side chain, by introducing bis-negatively charged functionalities (pK_{a1}/pK_{a2} values of malonate and monophosphate functions are 2.83/5.69, and 1.88/6.67, respectively, versus pK_a of 4−5 for the alkyl mono carboxylic function) for compounds **6** and **7**, extends and strengthens the hydrogen bond/electrostatic interactions with the Arg/Lys residues inside the nucleotide entry channel, leading to better stabilization of the dinucleoside phosphoramidate and to improved activity by blocking the access of nucleoside triphosphates (NTPs) to the polymerization site. In the case of the neutral side chain, the better activity observed for compound **8** as compared with compound **1** may be attributed to the greater length of the diethylene glycol methyl ether side chain of compound **8**, leading to greater obstruction for entering nucleotides and yet conserving a strong enough electronegative effect for favorable positioning inside this positively charged entry tunnel.[16]

Effect of 5′-Moiety for Compounds Bearing Charged Amino Side Chain on NS5B Polymerase Activity. Our previous SAR study showed that 5′-phosphate-bearing phosphoramidate dinucleosides were more potent inhibitors than the 5′-hydroxyl group equivalent ones. We then studied the influence of the nature of the 5′-moiety on the inhibitory effect of the 3′-H phosphoramidate dinucleosides bearing negatively charged amino side chains (Table 1). The phosphoramidate dinucleosides were synthesized either with a 5′-OH group (**3**, **4**, and **5**) or with a 5′-phosphate monoester (**2**, **6**, and **7**). As expected, the introduction of the phosphate monoester group at the 5′-position of **3**, **4**, and **5**, leading to compounds **6**, **7**, or **2**, respectively, resulted in a gain of activity up to 50%, with a decrease of IC_{50} from 21 (**3**) to 10 μM (**6**), from 19 (**4**) to 8 μM (**7**), from 67 (**5**) to 34 μM (**2**), confirming previous observations suggesting a higher affinity of monophosphate compounds for the active site.[29,30] Indeed, guanosine monophosphate (GMP) was previously shown to correctly initiate the RNA synthesis with a better efficiency than the natural guanosine triphosphate (GTP). These results are also consistent with our previously reported molecular model,[16] which showed that important hydrogen bond/electrostatic interactions between the 5′-phosphate moiety and three Arg residues (Arg158, Arg386, and Arg394) allowed better stabilization of the 5′-phosphorylated phosphoramidate dinucleosides.

Effect of Diastereoisomeric Mixture Separation on NS5B Polymerase Activity. Each synthesized phosphoramidate dinucleoside compound was obtained as a mixture of two

Table 2. Except for Compound **1**, Separation of Diastereoisomers Does Not Modify the Inhibition Potency of 3′-Deoxy Phosphoramidate Dinucleosides[a]

compd	IC_{50} (μM)
1-Rp	72 ± 25
1-Sp	7 ± 3
1′-Rp	28 ± 6
1′-Sp	37 ± 6
2-Rp	9 ± 1
2-Sp	13 ± 6
2′-Rp	26 ± 6
2′-Sp	16 ± 4
6-Rp	20.3 ± 1
6-Sp	18.5 ± 8
7-Rp	121 ± 11
7-Sp	57 ± 18
8-Rp	64 ± 19
8-Sp	42 ± 7

[a] IC_{50} values of separated diastereoisomers of neutral and negatively charged (**1**, **1′**, **2**, **2′**, **6**, **7**, and **8**) phosphoramidate dinucleosides. All data represent average values for at least three separated experiments.

diastereoisomers (Rp, Sp) on its internucleotidic chiral phosphorus center with ratio ranging from 1:1 to 1:2.7. Since all 5′-phosphate phosphoramidate dinucleosides were found more potent than the corresponding 5′-OH ones, we only chose to separate and evaluate in vitro the diastereoisomers of compounds **1**, **2**, **6**, **7**, and **8** (Table 2). In addition, GC 3′-OH compounds **1′** and **2′** were also separated and evaluated as referenced compounds (Table 2). Intriguingly, the Sp isomer of compound **1** bearing a neutral amino side chain exhibited the greatest inhibition potency (7 μM) of all compounds tested. Moreover, this isomer exhibited better inhibition potency than the Rp one and the corresponding mixture, displaying IC_{50} values 10-fold (72 μM, Table 2) and 4-fold (29 μM, Table 1) higher, respectively. This tendency was also observed with GC 3′-OH compound **1′** and **2′** but is less pronounced. Interestingly, this was not observed for compounds with negatively charged side chains (**2**, **6**, and **7**) as well as for compound bearing a larger neutral side chain (**8**). Indeed, IC_{50} values for both the mixtures on one hand, and either of the separated isomers, on the other, of compound **2**, **6**, **7**, and **8** were similar. Indeed, the Sp isomer in the 3′-OH series displayed a slightly better inhibition property. This difference between 3′-OH versus 3′-H strongly suggests that particularly the 3′-OH group of the Sp isomer interacts differently than the Rp one with amino acids of the catalytic site, explaining that in the absence of 3′-OH the observed effect is abolished.

Molecular Dynamics Study of the Polymerase in Complex with GC 3′-H Phosphoramidate Dinucleoside Bearing Methylamine Side Chain. In our previous study,[16] we used a model structure based on the analogy between HCV and bacteriophage RdRp's initiation complexes.[12,31,32] The structural alignment of bacteriophage and HCV RdRp's was produced using VMD. Subsequently, nucleic acids from bacteriophage RdRp were inserted into the HCV RdRp X-ray structure.[32] Two GTPs were then replaced by dinucleotides bearing the phosphoramidate linkages. Unfortunately, this previous model was not sufficient to account for the gain of activity conferred by 3′-H relative to 3′-OH phosphoramidate dinucleosides and also the difference of behavior between the diastereoisomers of GC 3′-H bearing either a neutral or a negatively charged side chain. A new model of HCV NS5B-GC 3′-H complex was thus based on the crystal structures of the Norwalk Virus RdRp complexed with nucleic acids as well as inhibitors which appeared in the literature very recently (PDB codes: 3BSN, 3BSO,[33] 3H5X, 3H5Y[34]). Indeed, the geometry of the active site of the Norwalk Virus RdRp was determined very precisely and the apparent structural similarity of Norwalk/HCV RdRps allowed straightforward transferability of many observations.

The stabilization of GC 3′-H dinucleoside phosphoramidate in the RdRp's active sites is mediated by mutual interplay (i) between dinucleosides side chains and positively charged Arg/Lys residues (Arg158, Arg48, Lys155) situated on the incoming pathway for nucleoside triphosphates (NTPs) into the active site, (ii) between the internucleotide linkage and Mg^{2+} ions, and (iii) thanks to hydrogen bonds, where 2′-OH and/or 3′-OH groups are involved (Figure 2).

The Sp and Rp diastereoisomers as well as their mixture in the case of compounds bearing a negative side chain (**2**, **6**, and **7**) displayed similar IC_{50} values. This feature may be explained by the interplay between negative side chains and positively charged Arg/Lys residues of the incoming NTPs channel dominating here and stabilizing firmly the dinucleosides in the active site of the enzyme as previously described.[16] Nevertheless, a slight difference between Sp and Rp isomers was observed in our previous SAR studies for GC 3′-OH compound bearing a carboxylic side chain and showing that the Sp isomer was more potent than the Rp one (see also Table 2, compound **2′**).

However, the most intriguing results were obtained here in the case of the Sp isomer of compound **1**, which exhibited a 9-fold better potency than its 3′-OH counterpart (7 vs 57 μM) and exhibited a 10-fold better potency than its Rp isomer. The short neutral side chain of compound **1** that was not able to interact significantly with basic Arg/Lys residues of the incoming NTPs channel, allowed probably to uncover Sp/Rp and 3′OH/3′H effects. Therefore, MD simulations were directed toward short neutral side chain to shed some light on these intriguing results.

The Sp isomer of GC 3′-H conserves a moderate stabilization owing to the nonbridging oxygen atom of the internucleosidic linkage directed toward Mg^{2+} ions that secure mutual interactions between internucleotide linkages and ions (Figure 2). Moreover, careful comparison with the reference simulation performed for Sp-GC 3′-OH uncovered that there are subtle differences considering the identity of the magnesium ion interacting with the nonbridging oxygen (see Figure 2 and D1, D2 graphs in Supporting Information). Interestingly, Sp-GC 3′-H was shifted toward the second Mg^{2+} ion within a MD run. In contrast, Sp-GC 3′-OH resided rather in the close proximity of the first Mg^{1+} ion. It should be noted, that Mg^{2+} can be usually found in the active sites of polymerases even lacking nucleic acids substrate. In contrast, Mg^{1+} is supposed to enter the active site as the accompanist of incoming NTPs and to leave it accompanying the pyrophosphate moiety released after nucleotide incorporation in growing RNA. Further, no stabilization was observed for Rp GC 3′-H oriented internucleotide linkages, which lost contacts with ions because the NH part of the side chain is directed toward Mg^{2+} ions (Figure 2 and D1, D2 graphs in Supporting Information).

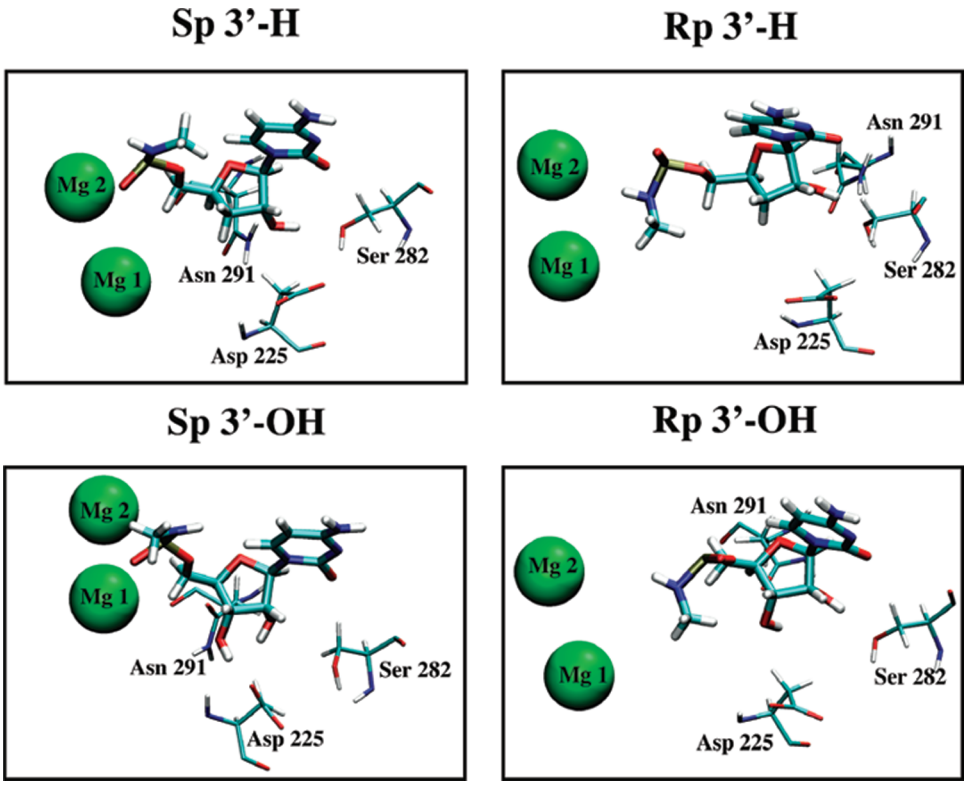


Figure 2. Molecular modeling of Sp/Rp GC 3′-H and Sp/Rp GC 3′-OH bearing a methylamino side chain and their interactions with amino acid residues of the NS5B polymerase active site (Asn291, Asp225, and Ser282) . Amino acid residues of NS5B polymerase active site are depicted as thin sticks. For clarity, only the side chain and terminal cytidine of the dinucleoside are depicted as thick sticks. Mg^{2+} ions are illustrated as two green spheres.

In summary, these data explain why GC 3′-H bearing a neutral side chain in the Sp absolute configuration, which is not able to interact with basic Arg/Lys residues of the NTPs channel, exhibited remarkably stronger inhibition activity (comparing either to the Sp-GC 3′-OH counterpart or both 3′-H and 3′-OH Rp isomers). This effect was not observed with compound bearing a larger neutral amino side chain as compound **8**. The difference observed for compound **8** may be attributed to the greater length of its diethylene glycol methyl ether side chain, leading to greater obstruction for entering nucleotides and yet conserving a strong enough electronegative effect for favorable positioning inside this positively charged entry tunnel.[16]

Differences in the recognition of the nonbridging oxygen from the phosphoramidate linkages (Sp-GC 3′-OH vs Sp-GC 3′-H) by either Mg^{2+} or Mg^{1+} ions are rooted in the hydrogen bonds connecting the 3′-OH group with the Asp225 residue (Figure 2, see D6−D7 graphs in Supporting Information). As a consequence, Sp-GC 3′-OH is repositioned slightly comparing to Sp-GC 3′-H leading to strengthening of contacts with Mg^{1+}, which offers less sound stabilization in the active site (comparing to Mg^{2+}).

Further, in the case of GC 3′-H series, Asp225 and Ser282 of HCV NS5B were connected by mutual hydrogen bonds (Figure 2, see D4−D5 graphs in Supporting Information) and the 2′-OH group of GC 3′-H series was recognized firmly by Ser282 (Figure 2, see D8 graphs in Supporting Information). In contrast, in the case of GC 3′-OH series, the 3′-OH group was competing with Ser282 and often replaced it in Asp225 binding, destabilizing 2′-OH interactions with Ser282. It could contribute further to a better stabilization of the 3′-H GC series than the GC 3′-OH series in the active site of HCV RdRp and therefore to a better inhibition activity.

Activity of Phosphoramidate Dinucleosides on HCV Subgenomic Replicon Replication. Target dinucleotides (**1**, **1-Sp**, **1-Rp**, **1′**, **1′-Sp**, **1′-Rp**, **2**, **6**, **7**, and **8**) were evaluated in Huh-6 cells, a hepatocyte cell line stably carrying a genotype 1b subgenomic HCV replicon. Replicon RNA levels were determined by a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay. The antiviral activity was then measured by the reduction in replicon RNA levels in the presence of increasing amounts of inhibitors. Concomitantly, the antimetabolic effect of each compound was evaluated as well. Calculated EC_{50} and CC_{50} values for evaluated compounds are summarized in Table 3. All compounds proved to be noncytotoxic, as no antimetabolic effect was detected at the highest concentration tested (65 μM) and exhibited particularly good inhibition of HCV replicon. As expected, activities for novel GC 3′-H series are improved compared to equivalent compounds of the GC 3′-OH series.[16]

As previously described, GC 3′-OH compound **1′** was inactive (Table 3). As expected, the GC 3′-H equivalent compound **1** was more active than the GC 3′-OH compound **1′** (19 μM vs > 50 μM). The separated **1′-Rp** and **1′-Sp** isomers, never tested before, were active and the Sp one was 8-fold more active than the Rp one (Table 3). As observed for compound **1′** isomers, separated isomers **1-Rp** and **1-Sp** were both significantly more potent than the mixture (8−12-fold). Surprisingly, **1-Rp** and **1-Sp** showed similar potencies (1.6 and 2.5 μM). These data were in contrast with the in vitro data obtained on purified NS5B, which showed a significant difference between both isomers (72 and 7 μM). This observation suggested that

Table 3. Antiviral Activity of Compounds **1**, **1-Sp**, **1-Rp**, **1′**, **1′-Sp**, **1′-Rp**, **2**, **6**, **7**, and **8** Was Evaluated at 10 Concentrations Ranging from 0.025 to 65 μM in Huh-6 Cells Containing a Subgenomic HCV Replicon (Genotype 1b)[a]

compd	EC_{50} (μM)	CC_{50} (μM)
1	19.4 ± 6.6	> 65
1-Rp	1.6 ± 0.3	> 65
1-Sp	2.5 ± 0.2	> 65
1′	> 50	> 65
1′-Rp	38 ± 7.2	> 65
1′-Sp	4.5 ± 0.5	> 65
2	1.7 ± 0.2	> 65
6	3.9 ± 2.0	> 65
7	2.6 ± 0.8	> 65
8	3.6 ± 0.4	> 65

[a] The antiviral activity is measured by the reduction in replicon RNA levels in the presence of the compound, taking into account that untreated replicon-containing cells produce the highest replicon RNA level (replicon control). Concomitantly with the evaluation of the antiviral activity of each compound, the antimetabolic effect is evaluated by means of the MTS/PMS method (see Experimental Section). EC_{50} values thus represent an average value of three independent experiments. Standard deviation values are provided as well.

the less active compound **1-Rp** on purified NS5B, could be converted in cell in something more active against HCV. Compounds **1** and **8**, bearing neutral amino side chains, resulted in a good inhibition of HCV replicon with EC_{50} values of 19.4 (shorter amino side chain) and 3.6 μM (longer amino side chain), respectively. Compounds **2**, **6**, and **7** in diastereoisomeric mixture, bearing negatively charged amino side chains, exhibited the strongest inhibition of HCV replicon with EC_{50} values of 1.7, 3.9, and 2.6 μM, respectively. No major difference is observed between mono- and dicarboxylic compounds, in contrast to the results of the in vitro evaluation.

Conclusion

In our search for new potent HCV polymerase inhibitors, we have synthesized a novel series of phosphoramidate dinucleosides named "GC 3′-H" based on our previous results[16] and evaluated their inhibitory effect on HCV NS5B polymerase in vitro and in HCV replicon-containing Huh-6 cells. The analogues of the present study harbor a 3′-deoxycytidine moiety introduced in order to enhance the inhibitory character as a chain terminator. As expected, all IC_{50} of GC-3′-H compounds were shown to be lower than those previously obtained with GC-3′-OH series (between 7 and 72 μM vs 33 up to > 300 μM).

A previous report showed that the only way to accommodate the long phosphoramidate side chain of GC 3′-OH series into the HCV RdRp active site was to direct it toward the nucleotide entry channel.[16] Thus, a negatively charged side chain could interact with the Lys/Arg residues of the incoming nucleotide channel, blocking the access of nucleotides to the polymerization site. Accordingly, the most potent inhibitors in vitro of the present study proved to be the 5′-phosphorylated GC 3′-H compounds bearing a bis-negatively charged side chain **6** (IC_{50} of 8 μM) and **7** (IC_{50} of 10 μM). However, the Sp isomer of a GC 3′-H compound bearing a neutral side chain **1** exhibited inhibitory activity in a close range of IC_{50} value (IC_{50} of 7 μM for the Sp isomer). To understand the role of the 3′ group of phosphoramidate dinucleoside, a new model of the HCV NS5B-GC 3′-H complex was refined by molecular dynamics simulations. This model revealed crucial interactions for inhibition between the 3′-group of phosphoramidate dinucleosides and amino acid residues within the catalytic site as well as Mg^{2+} ions.

The present GC 3′-H compounds showed increased activities in comparison to the GC 3′-OH ones, in HCV NS5B polymerase in vitro assays as well as in the HCV replicon system in cell culture. Indeed, all tested compounds proved to be noncytotoxic, as no antimetabolic effect was detected at the highest concentration tested (65 μM) and exhibited excellent inhibition of HCV replicon with EC_{50} value of 1.7 μM for the most potent one (compound **2**), bearing a negatively charged amino side chain. Novel negatively charged amino side chain introduced here showed excellent activity in a similar range, confirming and extending out previous report.[16] Nevertheless, negatively charged compounds, due to their polar nature, are known for not easily crossing lipid cell membranes. Therefore, decreasing the polar nature of our bis-negatively charged compounds by synthesis of equivalent prodrugs could enhance the activity of our hit compounds and make them new candidates for HCV therapy.

Experimental Section

General Procedures. General procedures have been given for the coupling, oxidation, and deprotection of target compounds. Variations from these procedures and individual purification methods are given in the main text for each compound. Experimental procedures for antiviral evaluation, general chemistry experimental and analysis conditions, preparative and spectroscopic data for the 3′-deoxycytidine monomer compounds **9** to **11**, for the amino intermediates **14**−**22**, for *H*-phosphonate diesters **23** and **24**, and full spectroscopic data for all target compounds (**1**−**8**) are given as Supporting Information only.

Oxidation Method A. In a dry flask, appropriate *H*-phosphonate diester (1 mol equiv) was dried by azeotropic distillation with anhydrous pyridine and dried under vacuum with P_2O_5 for 30 min. It was then dissolved in anhydrous pyridine (5 mL/mmol). To this solution were simultaneously added anhydrous CCl_4 (25 mol equiv, 2.5 mL/mmol) and corresponding amine (10 mol equiv) in anhydrous pyridine. The mixture was stirred at room temperature for 1 h. The solvents were removed under reduced pressure, and the residue was coevaporated twice with acetonitrile. Compounds were directly involved in deprotection steps.

Oxidation Method B. In a dry flask, appropriate *H*-phosphonate diester (1 mol equiv) was dried by azeotropic distillation with anhydrous pyridine and dried under vacuum with P_2O_5 for 30 min. It was then dissolved in anhydrous pyridine (2.5 mL/mmol). To this solution were simultaneously added anhydrous CCl_4 (25 mol equiv, 2.5 mL/mmol), 4 M solution of the corresponding amine hydrochloride or hydrotrifluoroacetate (10 mol equiv) in anhydrous pyridine, and anhydrous triethylamine (10 mol equiv). The mixture was stirred at room temperature for 1 h. The solvents were removed under reduced pressure, and the residue was coevaporated twice with acetonitrile. Compounds were directly involved in deprotection steps.

Deprotection Method A. Appropriate compound was dissolved in the corresponding solvent (14 mL/mmol), and 28% aqueous ammonia solution was added (70 mL/mmol). The flask was hermetically closed, and the mixture was stirred 7 h at 50 °C. The solvents were removed under reduced pressure to give the corresponding compound as brown oil, which was further purified by chromatography.

Deprotection Method B. Appropriate compound was dissolved in methanol (12 mL/mmol), and 80% acetic acid solution (62 mL/mmol) was added, then the mixture was stirred 2 h at room temperature. It was finally diluted with 10 mL of water and washed with dichloromethane (5 times) and ether (3 times). The aqueous layer was evaporated to dryness, and then the residue was purified by chromatography.

Deprotection Method C. Appropriate compound was dissolved in THF/acetonitrile 1:1 (v/v) (50 mL/mmol), and 0.4 M aqueous NaOH solution (100 mL/mmol) was added, then the

mixture was stirred at room temperature (reaction time is indicated for each compound in the main text). The reaction mixture was quenched with DOWEX-pyridinium resin. The resin was filtered off, and the filtrates were evaporated to dryness and coevaporated three times with ethanol, and then the residue was purified by chromatography.

HPLC Method for Separation of Diastereoisomers. Waters DeltaPak C_{18} preparative column was used on a Waters apparatus with a 2 mL/min flow rate (see Supporting Information). Gradient conditions, run times, and maximal loading of the column are given for each compound. After purification, the TEAAc buffer was removed after several lyophilizations from water. According to retention times on RP-HPLC the names "fast eluting" and "slow eluting" were attributed to each isomer.

HPLC Method for Purity Analysis. Nucleosil C18 column, (150 mm × 4.6 mm, 5 μm) from Supelco, Inc. was used on a Waters apparatus with a 1 mL/min flow rate (see Supporting Information Table S2). Two gradient conditions were used (see Supporting Information Table S2). Run times are given for each compound. According to retention times on RP-HPLC, the names "fast eluting" and "slow eluting" were attributed to each isomer. Purity of each compound is >95.8%, except for compound **7**slow, which is contaminated by 14% of **7**fast (see Supporting Information Figure S1).

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*N*-(2-methoxyethyl)-*O*-(2′-*O*-methyl-5′-*O*-phosphoryl guanosin-3′-yl) Phosphoramidate, Sodium Salt (1). Prepared according to oxidation method A from **24** (0.06 mmol, 1 equiv), 0.15 mL of CCl_4, 0.4 mL of pyridine, and 2-methoxyethylamine (60 μL, 0.7 mmol, 11 equiv), and according to deprotection method A using 1.5 mL of THF/MeOH 2:1 (v/v) and 4 mL of ammonia. Purified on preparative RP-HPLC (gradient: acetonitrile 6−11% in 30 min; maximal loading of the column: 10 mg of crude product per run). After elution on a DOWEX-Na^+ column and lyophilization from water, **1** was a white spongy solid (26 mg, 68%) epimeric mixture (ratio ∼ 1:1). The two diastereoisomers of the obtained mixture were separated by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 7−10% in 30 min; maximal loading of the column: 1 mg of mixture per run.

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*O*-(2′-*O*-methyl-5′-*O*-phosphorylguanosin-3′-yl)-*N*-(5-carboxypentyl) Phosphoramidate, Sodium Salt (2). Prepared according to oxidation method B, **23** (0.13 mmol, 1 equiv), 0.3 mL of CCl_4, 0.3 mL of pyridine, dry 6-aminocaproic acid methyl ester hydrochloride (236 mg, 1.3 mmol, 10 equiv) dissolved in 0.5 mL of pyridine and triethylamine (0.2 mL, 10 equiv), then according to deprotection method C using 5 mL of THF/ACN 1:1 (v/v) and 10 mL of 0.4 M NaOH solution after overnight stirring and according to deprotection method A using 10 mL of ammonia. Purified on a DEAE-A25 Sephadex column (gradient: TEAB 10^{-3} to 0.5 M) then by flash RP chromatography (gradient: acetonitrile 0−20%). After elution on a DOWEX-Na^+ column and lyophilization from water, **2** was a white spongy solid (30 mg, 30%) epimeric mixture (ratio ∼ 1:2.3).

The two diastereoisomers of the obtained mixture were separated by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 6−8% in 40 min; maximal loading of the column: 1.2 mg of mixture per run.

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*O*-(2′-*O*-methylguanosin-3′-yl)-*N*-(3-phosphoryloxy propyl) Phosphoramidate, Sodium Salt (3). Prepared according to oxidation method B from **24** (0.04 mmol, 1 equiv), 0.1 mL of CCl_4, 0.1 mL of pyridine, dry **16** (78 mg, 0.2 mmol, 5 equiv) dissolved in 0.1 mL of pyridine and triethylamine (0.1 mL, 10 equiv), then according to deprotection method A using 2.5 mL of THF/ACN 1:1 (v/v) and 5 mL of ammonia, and according to deprotection method B using 5 mL of 80% acetic acid. Purified on a DEAE-A25 Sephadex column (gradient: TEAB 10^{-3} to 0.1 M) then by flash RP chromatography (gradient: acetonitrile 0−20%). After elution on a DOWEX-Na^+ column and lyophilization from water, **3** was a white spongy solid (9.4 mg, 30%) epimeric mixture (ratio ∼ 1:1.5).

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*O*-(2′-*O*-methylguanosin-3′-yl)-*N*-[3-(malon-2-yl) propyl] Phosphoramidate, Sodium Salt (4). Prepared according to oxidation method B from **24** (0.04 mmol, 1 equiv), 0.1 mL of CCl_4, 0.1 mL of pyridine, dry **19** (130 mg, 0.4 mmol, 10 equiv) dissolved in 0.1 mL of pyridine and triethylamine (0.1 mL, 10 equiv), then according to deprotection method C using 2.5 mL of THF/ACN 1:1 (v/v) and 7 mL of 0.4 M NaOH solution after overnight stirring, then according to deprotection method A, using 5 mL of ammonia, and finally according to deprotection method B using 5 mL of 80% acetic acid. Purified on a DEAE-A25 Sephadex column (gradient: TEAB 10^{-3} to 0.1 M) then by flash RP chromatography (gradient: acetonitrile 0−20%). After elution on a DOWEX-Na^+ column and lyophilization from water, **4** was a white spongy solid (10 mg, 32%) epimeric mixture (ratio ∼ 1:1.5).

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*O*-(2′-*O*-methylguanosin-3′-yl)-*N*-(5-carboxypentyl) Pphosphoramidate, Sodium Salt (5). Prepared according to oxidation method B from **24** (0.04 mmol, 1 equiv), 0.1 mL of CCl_4, 0.1 mL of pyridine, dry 6-aminocaproic acid methyl ester hydrochloride (100 mg, 0.4 mmol, 10 equiv) dissolved in 0.1 mL of pyridine and triethylamine (0.1 mL, 10 equiv), then according to deprotection method C using 2.5 mL of THF/ACN 1:1 (v/v) and 5 mL of 0.4 M NaOH solution after 2 h stirring, then according to deprotection method A, using 5 mL of ammonia, and finally according to deprotection method B using 5 mL of 80% acetic acid. Purified by flash RP chromatography (gradient: acetonitrile 0−10%). After elution on a DOWEX-Na^+ column and lyophilization from water, **5** was a white spongy solid (14 mg, 50%) epimeric mixture (ratio ∼ 1:2.7).

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*O*-(2′-*O*-methyl-5′-*O*-phosphorylguanosin-3′-yl)-*N*-(3-phosphoryloxypropyl) Phosphoramidate, Sodium Salt (6). Prepared according to oxidation method B from **23** (0.06 mmol, 1 equiv), 0.2 mL of CCl_4, 0.2 mL of pyridine, dry **16** (300 mg, 0.7 mmol, 13 equiv) dissolved in 0.2 mL of pyridine and triethylamine (0.2 mL, 10 equiv), then according to deprotection method A using 2.5 mL of THF/EtOH 1:2 (v/v) and 10 mL of ammonia after 10 h stirring. Purified on a DEAE-A25 Sephadex column (gradient: TEAB 10^{-3} to 0.3 M) then by flash RP chromatography (gradient: acetonitrile 0−50%). After elution on a DOWEX-Na^+ column and lyophilization from water, **6** was a white spongy solid (10 mg, 19%) epimeric mixture (ratio ∼ 1:1.3). One mg of the diastereoisomeric mixture was separated by analytical RP-HPLC using the general conditions described above. Gradient: acetonitrile 6−8% in 40 min.

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*O*-(2′-*O*-methyl-5′-*O*-phosphorylguanosin-3′-yl)-*N*-[3-(malon-2-yl)propyl] Phosphoramidate, Sodium Salt (7). Prepared according to oxidation method B from **23** (0.07 mmol, 1 equiv), 0.2 mL of CCl_4, 0.2 mL of pyridine, dry **19** (250 mg, 0.7 mmol, 10 equiv) dissolved in 0.3 mL of pyridine and triethylamine (0.2 mL, 10 equiv), then according to deprotection method C using 2.5 mL of THF/ACN 1:1 (v/v) and 7 mL of 0.4 M NaOH solution after overnight stirring, and according to deprotection method A, using 5 mL of ammonia. Purified on a DEAE-A25 Sephadex column (gradient: TEAB 10^{-3} to 0.3 M) then by flash RP chromatography (gradient: acetonitrile 0−50%). After elution on a DOWEX-Na^+ column and lyophilization from water, **7** was a white spongy solid (13 mg, 21%) epimeric mixture (ratio ∼ 1:2.3).

The two diastereoisomers of the obtained mixture were separated by preparative RP-HPLC using the general conditions described above. Gradient: acetonitrile 3−6% in 45 min; maximal loading of the column: 1 mg of mixture per run.

Synthesis of *O*-(3′-Deoxycytidin-5′-yl)-*N*-[2-(2-methoxyethoxy)-ethyl]-*O*-(2′-*O*-methyl-5′-*O*-phosphorylguanosin-3′-yl) Phosphoramidate, Sodium Salt (8). Prepared according to oxidation method A from **23** (0.05 mmol, 1 equiv), 0.2 mL of CCl_4, 0.2 mL of pyridine and dry **22** (80 mg, 0.6 mmol, 12 equiv) dissolved in 0.2 mL of pyridine, and according to deprotection method A using 1.5 mL of THF/MeOH 2:1 (v/v) and 5 mL of ammonia. Purified on a

DEAE-A25 Sephadex column (gradient: TEAB 10^{-3} to 0.3 M) then by flash RP chromatography (gradient: acetonitrile 0−50%). After elution on a DOWEX-Na^+ column and lyophilization from water, **8** was a white spongy solid (23 mg, 57%) epimeric mixture (ratio ~1:1.4).

HCV 1b Polymerase Preparation and Reagents. NS5B-D55 gene, tagged with six C-terminal histidines was expressed from pDest 14 vector (Invitrogen) in *Escherichia coli* BL21(DE3) cells (Novagen) and was purified as previously described.[16] RNA H(−) ($^{5'}$-UCGGGGGCUGGC-$^{3'}$) oligonucleotides was obtained from MWG-Biotech. a-^{32}P-labeled cytosine 5′-triphosphate (3000 Ci/mmol) was purchased from Amersham.

Determination of Inhibitory Concentration 50% (IC_{50}). The RNA oligonucleotide (RNA H (−)) corresponding to the 3′ end of the negative strand of the HCV genome was used to analyze the synthesis of the first phosphodiester bond and its inhibition induced by the modified phosphoramidate dinucleotides. The dinucleotides concentration leading to 50% inhibition of NS5B-mediated RNA synthesis was determined in RdRp buffer (50 mM HEPES pH 8.0, 10 mM KCl, 10 mM DTT, 0.5% Igepal CA630) containing 10 mM of RNA H(−) template, 1 μM NS5B and various concentration of dinucleotides (0, 0.01, 0.1, 1, 10, 50, 100, 1000 μM). Reactions were initiated by the addition of 2 mM $MnCl_2$, 1 mM $MgCl_2$, 50 μM γ-^{32}P-GTP (1 μCi), and 100 μM CTP. After 15 min incubation at 30 °C, samples were quenched with EDTA/formamide. Products were separated using sequencing gel electrophoresis and quantified using photostimulated plates and a FujiImager (Fuji). IC_{50} was determined using the equation: % of active enzyme = $100/(1 + (I)^2/IC_{50})$, where I is the concentration of the inhibitor. IC_{50} was determined from curve-fitting using Kaleidagraph (Synergy Software).

Molecular Dynamics. The model of the HCV RdRp-GC 3′-H complex is based on the analogy with the crystal structure of the Norwalk Virus RdRp complexed with nucleic acids as well as inhibitors which appeared in literature very recently (3BSN, 3BSO,[33] 3H5X, 3H5Y[34]). The geometry of the active site was determined very precisely in 3BSO.pdb,[33] uncovering clearly certain rearrangements of hydrogen bonding connecting the 2′-OH/3′-OH groups of GC dinucleosides with Asp247, Ser300, and Asn309 of the Norwalk polymerase (their counterparts in HCV RdRp were Asp225, Ser282, and Asn291). There is an apparent structural similarity of Norwalk/HCV RdRps, which allow straightforward transferability of many observations. The pyrophosphate moiety from CTP in 3BSO.pdb was deleted and the rC nucleotide was simply connected to the 3′-terminus of the RNA transcript. We supposed that positions of the GpC dinucleotide in the initiation complex and of the relevant part of the RNA transcript in the elongation complex differ only negligibly.

Molecular dynamics simulations were produced for six simulated systems differing mutually in the rC residue modifications. There were used (i) natural rC, (ii) 3′-H rC, (iii, v) natural rC bearing either Sp or Rp oriented phosphoramidate linkages, and (iv,vi) 3′-H rC with Sp/Rp phosphoramidate linkages. Phosphoramidate linkages were represented by a simplified P-$NHCH_3$ variant. Longer side-chains should interact with either positively charged residues or magnesium ions as proposed previously.[16] This required necessary completion and modification of all_nuc94.in (molecular topology) and parm94.dat (force field) files. Several bond (NS_P) angle and dihedral angle (CT-NS-P-OS, X-CT-NS-P) terms were taken from ref 35 in which the AMBER parameters for a similar internucleotide linkage modification were developed. Remaining charges, as well as bond, angle, and dihedral terms for the side chains, were taken from the AMBER database44 and did not require modification. Such approximation was judged sufficient for the geometrical factors analyzed here.

Simulated system was surrounded by ~15565 TIP3P water molecules[36] which extended to a distance of approximately 10 Å (in each direction) from the RdRp atoms. This gives a periodic box size of ~90 Å, ~97 Å, ~78 Å. New *.inpcrd (initial coordinates) and *.prmtop (molecular topology, force field etc.) files for the whole simulated system including modified residues were created by use of the TLEAP module (AMBER software package[37]).

Fully solvated trajectories (lasting for 5 ns) were computed with the aid of the NAMD software package.[38] Conventional computational procedures were used: periodic boundary conditions, cut off distances of 10 Å for the nonbonded interactions, and the particle-mesh-Ewald method for the summation of the Coulombic interactions.[39] MD time step = 0.002 ps. Initially, for 5 ps, the system was heated up to 310 K using a Langevin temperature equilibration scheme while restraining the position of the solute. The MD was then continued for 5 ns at constant T and constant P with all restraints removed.

Figures were produced with the aid of the VMD software package.[40]

Evaluation of Antiviral and Cytostatic Activities of Compounds in HCV Genotype 1b Subgenomic Replicon Carrying Huh-6 cells. Huh-6 cells containing genotype 1b subgenomic HCV replicon derived from the Con1 isolate were described elsewhere.[41] Huh-6 cells were seeded at a density of 1.5×10^4 cells per well in 96-well cell culture plates, and antiviral assays were carried out as described before.[41] Following incubation of 24 h at 37 °C (5% CO_2), serial dilutions ranging from 0.025 to 65 μM of the test compounds in complete DMEM were added in a total volume of 100 μL. After a 3-day incubation period at 37 °C (5% CO_2), cells were lysed in cells-to-cDNA lysis buffer (Ambion, Cambridgeshire, UK), and lysates were used to determine the amount of HCV replicon RNA by means of quantitative real-time PCR as described previously.[42] The EC_{50}s were calculated as the concentration of compound that caused a 50% reduction in HCV RNA levels compared to that of the untreated control. Serial dilutions of known quantities of a plasmid containing the neomycin gene were used to set up the standard curve. The amount of viral RNA produced in treated cultures was expressed as a percentage of that in the untreated control. To determine the cytotoxic activity of the compounds, an assay was set up in the same way described above, but after 3 days the cell number was determined by means of the MTS/PMS method (Promega) as described previously.[41] The CC_{50}s were calculated as the concentration of compound that caused a 50% reduction of the proliferation of exponentially growing replicon cells compared to that of the untreated control.

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Supporting Information Available: General experimental procedures, preparative and spectroscopic data for compounds **9**−**11** and **14**−**24** and full spectroscopic data for all target compounds; correlation table between HPLC retention time, δ ^{31}P NMR values, and suggested absolute configuration for separated isomers of compounds **1**, **2**, and **7**; purity data from HPLC analysis, ^{1}H NMR and ^{31}P NMR spectra for all target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) WHO. Hepatitis C—Global Prevalence (Update). *Wkly. Epidemiol. Rec.* **2000**, *75*, 18−19.
(2) Choo, Q. L.; Kuo, G.; Weiner, A. J.; Overby, L. R.; Bradley, D. W.; Houghton, M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **1989**, *244* (4902), 359–362.

(3) Kuo, G.; Choo, Q. L.; Alter, H. J.; Gitnick, G. L.; Redeker, A. G.; Purcell, R. H.; Miyamura, T.; Dienstag, J. L.; Alter, M. J.; Stevens, C. E.; et al. An assay for circulating antibodies to a major etiologic virus of human non-A, non-B hepatitis. *Science* **1989**, *244* (4902), 362–364.
(4) Takamizawa, A.; Mori, C.; Fuke, I.; Manabe, S.; Murakami, S.; Fujita, J.; Onishi, E.; Andoh, T.; Yoshida, I.; Okayama, H. Structure and organization of the hepatitis C virus genome isolated from human carriers. *J. Virol.* **1991**, *65* (3), 1105–1113.
(5) Moradpour, D.; Penin, F.; Rice, C. M. Replication of hepatitis C virus. *Nature Rev. Microbiol.* **2007**, *5* (6), 453–463.
(6) Suzuki, T.; Ishii, K.; Aizaki, H.; Wakita, T. Hepatitis C viral life cycle. *Adv. Drug Delivery Rev.* **2007**, *59* (12), 1200–1212.
(7) Pawlotsky, J. M.; Chevaliez, S.; McHutchison, J. G. The hepatitis C virus life cycle as a target for new antiviral therapies. *Gastroenterology* **2007**, *132* (5), 1979–1998.
(8) Manns, M. P.; McHutchison, J. G.; Gordon, S. C.; Rustgi, V. K.; Shiffman, M.; Reindollar, R.; Goodman, Z. D.; Koury, K.; Ling, M.; Albrecht, J. K. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **2001**, *358* (9286), 958–965.
(9) Manns, M. P.; W., H; Cornberg, M. Treating viral hepatitis C: efficacy, side effects, and complications. *Gut* **2006**, *55*, 1350–1359.
(10) Moriishi, K.; Matsuura, Y. Evaluation systems for anti-HCV drugs. *Adv. Drug Delivery Rev.* **2007**, *59* (12), 1213–1221.
(11) Major, M. E.; Dahari, H.; Mihalik, K.; Puig, M.; Rice, C. M.; Neumann, A. U.; Feinstone, S. M. Hepatitis C virus kinetics and host responses associated with disease and outcome of infection in chimpanzees. *Hepatology* **2004**, *39* (6), 1709–1720.
(12) Migliaccio, G.; Tomassini, J. E.; Carroll, S. S.; Tomei, L.; Altamura, S.; Bhat, B.; Bartholomew, L.; Bosserman, M. R.; Ceccacci, A.; Colwell, L. F.; Cortese, R.; De Francesco, R.; Eldrup, A. B.; Getty, K. L.; Hou, X. S.; LaFemina, R. L.; Ludmerer, S. W.; MacCoss, M.; McMasters, D. R.; Stahlhut, M. W.; Olsen, D. B.; Hazuda, D. J.; Flores, O. A. Characterization of resistance to non-obligate chain-terminating ribonucleoside analogs that inhibit hepatitis C virus replication in vitro. *J. Biol. Chem.* **2003**, *278* (49), 49164–49170.
(13) Asselah, T.; Benhamou, Y.; Marcellin, P. Protease and polymerase inhibitors for the treatment of hepatitis C. *Liver Int.* **2009**, *29* (Suppl 1), 57–67.
(14) Beaulieu, P. L. Recent advances in the development of NS5B polymerase inhibitors for the treatment of hepatitis C virus infection. *Expert Opin. Ther. Pat.* **2009**, *19*, 145–164.
(15) Kronenberger, B.; Zeuzem, S. Current and future treatment options for HCV. *Ann. Hepatol.* **2009**, *8*, 103–112.
(16) Zlatev, I.; Dutartre, H.; Barvik, I.; Neyts, J.; Canard, B.; Vasseur, J. J.; Alvarez, K.; Morvan, F. Phosphoramidate dinucleosides as hepatitis C virus polymerase inhibitors. *J. Med. Chem.* **2008**, *51* (18), 5745–5757.
(17) Zhong, W.; An, H.; Barawkar, D.; Hong, Z. Dinucleotide analogues as novel inhibitors of RNA-dependent RNA polymerase of hepatitis C Virus. *Antimicrob. Agents Chemother.* **2003**, *47* (8), 2674–2681.
(18) Zlatev, I.; Kato, Y.; Meyer, A.; Vasseur, J.-J.; Morvan, F. Use of a solid-supported coupling reagent for a selective phosphitylation of the primary alcohol of *N*2-isobutyryl-2′-deoxy or 2′-*O*-methyl guanosine. *Tetrahedron Lett.* **2006**, *47* (47), 8379–8382.
(19) Zlatev, I.; Vasseur, J.-J.; Morvan, F. Convenient synthesis of *N*2-isobutyryl-2′-*O*-methyl guanosine by efficient alkylation of *O*6-trimethylsilylethyl-3′,5′-di-*tert*-butylsilanediyl guanosine. *Tetrahedron* **2007**, *63* (45), 11174–11178.
(20) Zlatev, I.; Vasseur, J.-J.; Morvan, F. Deoxygenation of 5-*O*-benzoyl-1,2-isopropylidene-3-*O*-imidazolylthiocarbonyl-[alpha]-D-xylofuranose using dimethyl phosphite: an efficient alternate method towards a 3′-deoxynucleoside glycosyl donor. *Tetrahedron Lett.* **2008**, *49* (20), 3288–3290.
(21) Bhat, V.; Ugarkar, B. G.; Sayeed, V. A.; Grimm, K.; Kosora, N.; Domenico, P. A.; Stocker, E. A Simple and Convenient Method for the Selective N-Acylations of Cytosine Nucleosides. *Nucleosides Nucleotides* **1989**, *8* (2), 179–183.
(22) Jankowska, J.; Sobkowski, M.; Stawinski, J.; Kraszewski, A. Studies on Aryl H-Phosphonates 0.1. An Efficient Method for the Preparation of Deoxyribonucleoside and Ribonucleoside 3′-H-Phosphonate Monoesters by Transesterification of Diphenyl *H*-Phosphonate. *Tetrahedron Lett.* **1994**, *35* (20), 3355–3358.
(23) Adamo, I.; Dueymes, U.; Schonberger, A.; Navarro, A. E.; Meyer, A.; Lange, M.; Imbach, J. L.; Link, F.; Morvan, F.; Vasseur, J. J. Solution-phase synthesis of phosphorothioate oligonucleotides using a solid-supported acyl chloride with *H*-phosphonate chemistry. *Eur. J. Org. Chem.* **2006**, *2*, 436–448.
(24) Rachele, J. R. Methyl Esterification of Amino Acids with 2,2-Dimethoxypropane and Aqueous Hydrogen Chloride. *J. Org. Chem.* **1963**, *28* (10), 2898–2898.
(25) Zlatev, I.; Giraut, A.; Morvan, F.; Herdewijn, P.; Vasseur, J. J. delta-Di-carboxybutyl phosphoramidate of 2′-deoxycytidine-5′-monophosphate as substrate for DNA polymerization by HIV-1 reverse transcriptase. *Bioorg. Med. Chem.* **2009**, *17* (19), 7008–7014.
(26) Neumayer, D. A.; Belot, J. A.; Feezel, R. L.; Reedy, C.; Stern, C. L.; Marks, T. J. Approaches to alkaline earth metal-organic chemical vapor deposition precursors. Synthesis and characterization of barium fluoro-beta-ketoiminate complexes having appended polyether "lariats". *Inorg. Chem.* **1998**, *37* (21), 5625–5633.
(27) Tomoskozi, I.; Gacs-Baitz, E.; Otvos, L. Stereospecific conversion of H-phosphonates into phosphoramidates. The use of vicinal carbon-phosphorus couplings for configurational determination of phosphorus. *Tetrahedron* **1995**, *51* (24), 6797–6804.
(28) Zlatev, I.; Dutartre, H.; Barvik, I.; Neyts, J.; Canard, B.; Vasseur, J. J.; Alvarez, K.; Morvan, F. Phosphoramidate Dinucleosides as Hepatitis C Virus Polymerase Inhibitors. *J. Med. Chem.* **2008**, *51* (18), 5745–5757.
(29) Shim, J. H.; Larson, G.; Wu, J. Z.; Hong, Z. Selection of 3′-template bases and initiating nucleotides by hepatitis C virus NS5B RNA-dependent RNA polymerase. *J. Virol.* **2002**, *76* (14), 7030–7039.
(30) Ranjith-Kumar, C. T.; Gutshall, L.; Kim, M. J.; Sarisky, R. T.; Kao, C. C. Requirements for de novo initiation of RNA synthesis by recombinant flaviviral RNA-dependent RNA polymerases. *J. Virol.* **2002**, *76* (24), 12526–12536.
(31) Butcher, S. J.; Grimes, J. M.; Makeyev, E. V.; Bamford, D. H.; Stuart, D. I. A mechanism for initiating RNA-dependent RNA polymerization. *Nature* **2001**, *410* (6825), 235–240.
(32) Bressanelli, S.; Tomei, L.; Rey, F. A.; De Francesco, R. Structural analysis of the hepatitis C virus RNA polymerase in complex with ribonucleotides. *J. Virol.* **2002**, *76* (7), 3482–3492.
(33) Zamyatkin, D. F.; Parra, F.; Alonso, J. M.; Harki, D. A.; Peterson, B. R.; Grochulski, P.; Ng, K. K. Structural insights into mechanisms of catalysis and inhibition in Norwalk virus polymerase. *J. Biol. Chem.* **2008**, *283* (12), 7705–7712.
(34) Zamyatkin, D. F.; Parra, F.; Machin, A.; Grochulski, P.; Ng, K. K. Binding of 2′-amino-2′-deoxycytidine-5′-triphosphate to norovirus polymerase induces rearrangement of the active site. *J. Mol. Biol.* **2009**, *390* (1), 10–16.
(35) Cieplak, P.; Cheatham, T. E.; Kollman, P. A. Molecular dynamics simulations find that 3′ phosphoramidate modified DNA duplexes undergo a B to A transition and normal DNA duplexes an A to B transition. *J. Am. Chem. Soc.* **1997**, *119* (29), 6722–6730.
(36) Jorgensen, W. L.; Chandrasekhar, J.; Madura, J. D.; Impey, R. W.; Klein, M. L. Comparison of Simple Potential Functions for Simulating Liquid Water. *J. Chem. Phys.* **1983**, *79* (2), 926–935.
(37) Pearlman, D. A.; Case, D. A.; Caldwell, J. W.; Ross, W. S.; Cheatham, T. E.; Debolt, S.; Ferguson, D.; Seibel, G.; Kollman, P. Amber, a Package of Computer-Programs for Applying Molecular Mechanics, Normal-Mode Analysis, Molecular-Dynamics and Free-Energy Calculations to Simulate the Structural and Energetic Properties of Molecules. *Comput. Phys. Commun.* **1995**, *91* (1−3), 1–41.
(38) Phillips, J. C.; Braun, R.; Wang, W.; Gumbart, J.; Tajkhorshid, E.; Villa, E.; Chipot, C.; Skeel, R. D.; Kale, L.; Schulten, K. Scalable molecular dynamics with NAMD. *J. Comput. Chem.* **2005**, *26* (16), 1781–1802.
(39) Cheatham, T. E.; Miller, J. L.; Fox, T.; Darden, T. A.; Kollman, P. A. Molecular-Dynamics Simulations on Solvated Biomolecular Systems—the Particle Mesh Ewald Method Leads to Stable Trajectories of DNA, RNA and Proteins. *J. Am. Chem. Soc.* **1995**, *117* (14), 4193–4194.
(40) Humphrey, W.; Dalke, A.; Schulten, K. VMD: Visual molecular dynamics. *J. Mol. Graphics* **1996**, *14* (1), 33–38.
(41) Coelmont, L.; Paeshuyse, J.; Windisch, M. P.; De Clercq, E.; Bartenschlager, R.; Neyts, J. Ribavirin antagonizes the in vitro anti-hepatitis C virus activity of 2′-C-methylcytidine, the active component of valopicitabine. *Antimicrob. Agents Chemother.* **2006**, *50* (10), 3444–3446.
(42) Paeshuyse, J.; Kaul, A.; De Clercq, E.; Rosenwirth, B.; Dumont, J. M.; Scalfaro, P.; Bartenschlager, R.; Neyts, J. The non-immunosuppressive cyclosporin DEBIO-025 is a potent inhibitor of hepatitis C virus replication in vitro. *Hepatology* **2006**, *43* (4), 761–770.