HCV NS5b RNA-Dependent RNA Polymerase Inhibitors: From α,γ-Diketoacids to 4,5-Dihydroxypyrimidine- or 3-Methyl-5-hydroxypyrimidinonecarboxylic Acids. Design and Synthesis

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Abstract: A new class of the HCV NS5b RNA-dependent RNA polymerase inhibitors, the dihyroxypyrimidinecarboxylic acid derivative, was designed from a diketoacid and meconic acid derivative discovered by screening. Mechanism of action and essential moieties required for activity were identified. The corresponding *N*-methylpyrimidinone was also prepared; both classes are novel, reversible, and selective inhibitors of the HCV NS5b polymerase with improved druglike characteristics.

Hepatitis C virus (HCV) was identified in 1989 as the pathogen responsible for non-A and non-B hepatitis (NANB-H).[1] It has been estimated that 1−3% of the world population is infected. Seventy to eighty percent of infections become chronic and may progress to cirrhosis. In addition, increased incidence of hepatocellular carcinoma in NANB-H patients suggests that HCV plays a role in the progression of hepatocarcinogenesis. The recommended therapies are based on interferon-α (IFN-α) alone or in combination with the broad spectrum antiviral ribavirin. Only in a fraction of the patient population are these therapies effective.[2,3] They also cause severe side effects that significantly reduce compliance to the therapy. There is thus an obvious need to develop an efficacious and well tolerated anti HCV agent.[4,5]

HCV is a member of the flaviviridae viruses and is a small enveloped positive-stranded RNA virus that encodes a polyprotein of 3000 amino acids divided into four structural and six nonstructural proteins.[6] The HCV NS5b RNA-dependent RNA polymerase is a pivotal enzyme in the replication of the virus. Therefore, NS5b has become a prime target for both the screening and the design of small molecules inhibitors of viral replication. Recently, different classes of NS5b inhibitors have appeared in the literature: they can be divided by their mechanism of action into three major classes:[7] nucleoside,[8,9] non-nucleoside inhibitors acting at allosteric binding sites,[10–17] and last non-nucleoside inhibitors capable of interacting with the pyrophosphate binding site discovered in this laboratory.

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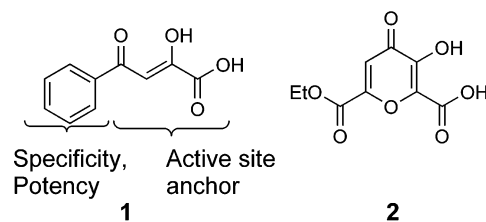
Figure 1. Leads for HCV NS5b polymerase.

Recently, we reported the discovery by random screening of α,γ-diketoacid **1** (DKA) as potent, reversible, and selective inhibitors of HCV NS5b RNA-dependent RNA polymerase with $IC_{50} = 5.7$ μM.[18] The α,γ-diketoacid functionality proved to be essential for enzyme inhibition in this class of inhibitors. It can be considered as an active site anchor because it interacts with the Mg^{2+} ions, i.e., the catalytic center of the polymerase. The aromatic portion of the molecules proved to be essential for specificity and potency (Figures 1, **1**).

In a second random screening the monoethylester of meconic acid **2** (Figure 1, **2**) was discovered as a new class of selective and reversible inhibitor of the HCV NS5b polymerase, having an $IC_{50} = 2.25$ μM.[19]

The two screening leads share a very similar chelating portion formed by a carbonyl, one acid hydroxyl function, and a carboxylic acid. A displacement competition experiment between the DKA **1** and the meconic acid derivative **2** indicated that the two compounds inhibit the enzyme in a mutually exclusive fashion, indicating a common mechanism of action. Kinetic competition experiments confirmed this result. However, there was concern for the development of these compounds as chemotherapeutic agents due to their intrinsic chemical and biological instability.[20] Therefore, we sought viable replacements of these core structures with more druglike characteristics. Several possible classes of inhibitors were designed by keeping the distances fixed between the chelating functions and the other moieties that generate the specificity. Particularly useful in assigning the correct distances between the different atoms that should interact with the metals in the active site was the X-ray structure of the T7 DNA polymerase cocrystallized with a nucleoside triphosphate chelating the two metals.[21] The distance between the two oxygens of the triphosphate engaged in a Mg^{2+} chelation is 3.1 Å. In the CSD is also available the crystal structure of a diketo compound chelating Mg^{2+} with the distance between the two oxygens being 2.8 Å. On the basis of these distances, several possible chelation patterns were considered, and some of them were prepared and tested without success. However, the dihydroxypyrimidinecarboxylic acid **3** designed as hybrid of DKA **1** and meconic acid derivatives **2** (Figure 2) was particularly attractive.

Superimposition of DKA **1** and dihydroxypyrimidinecarboxylic acid **3** shows a good overlap of the chelating moieties and the aromatic ring of the two inhibitors (Figure 3, A). Even better overlap of the chelating moiety was obtained with the meconic acid derivative **2** and **3** (Figure 3, B).

An aryl moiety was preferred to the carboxylic ester as a fragment to insert in the hybrid to improve the
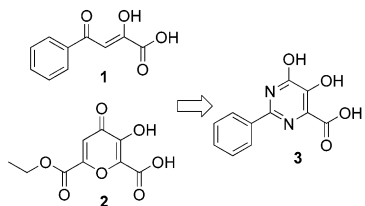
Figure 2. Design of dihydroypyrimidinecarboxylic acid derivative **3** from **1** and **2**.
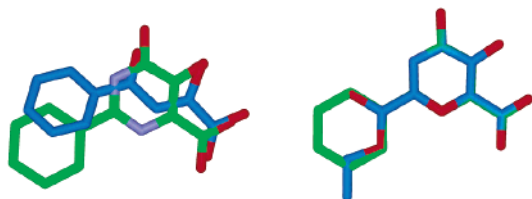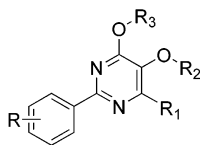

Figure 3. (A) Superimposition of dihydroxypyrimidine (green) and DKA **1** (blue). (B) Superimposition of dihydroxypyrimidine and meconic acid derivative **2** (blue).

Table 1.

compd	R	R_1	R_2	R_3	IC_{50} Mg/μM^a	IC_{50} Mn/μM^a
3	H	CO_2H	H	H	30	2.3
4	4-Cl	CO_2Me	H	H	>100	30
5	4-Cl	CO_2H	H	H	29	1.4
6	3-OBn	CO_2H	H	H	15	1.0
7	3-OH	CO_2H	H	H	5.8	0.6
8	3-OH	CO_2H	H	Me	>50	>50
9	4-Cl	CO_2H	Me	H	>50	>50
10	3-OH	H	H	H	>50	>50

^{a} Polymerase assay: see Supporting Information.

druglike character of the new molecules. Searching commercially available samples, we found the methyl dihydroxypyrimidinecarboxylic ester derivative **4** (Table 1) that corresponded very well to our designed molecule. This compound under our routine screening conditions resulted inactive at 100 μM in the presence of Mg^{2+} but showed an $IC_{50} = 30$ μM in the presence of Mn^{2+} (Table 1). These results indicated that **4** was capable of inhibiting the HCV NS5b polymerase. The methyl ester of **4** was hydrolyzed to corresponding acid **5** that was active both in the presence of Mg^{2+} and Mn^{2+} showing IC_{50}'s of 29 μM and 1.4 μM, respectively (Table 1). The biochemical behavior of this new class of inhibitors was identical to the other leads **1** and **2**, showing an increasing inhibitory potential in the presence of Mn^{2+} with respect to Mg^{2+}. Kinetic competition experiments between the DKA **1** and the new lead **5** confirmed a mutually exclusive inhibition.

The unsubstituted compound **3**, prepared as a reference compound, was equipotent to the lead **5** under both assay conditions (Mg^{2+} or Mn^{2+}), indicating that an electron-withdrawing group in the para position of the aryl moiety is a neutral substitution.

On the basis of the experience with the DKAs, in which a benzyl ether or a simple phenolic function enhanced the inhibitory activity with respect to the original DKA **1**, these two moieties were incorporated
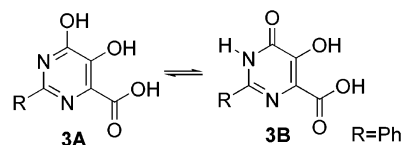

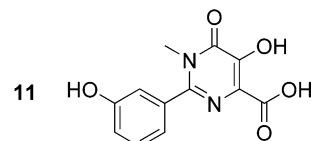**Figure 4.** Two of the possible tautomers of the dihyroxypyrimidine **3**.

Figure 5. *N*-Methylpyrimidinone **11**.

on the phenyl ring of **3**. The benzyl ether moiety was introduced in all three aromatic positions. While the *o*- and *p*-benzyl ether-substituted analogues **3** led to inactive compounds (data not shown), the meta-substituted benzyl ether derivative **6** gave a compound 2-fold more potent showing an $IC_{50} = 15$ μM (Mg^{2+}). **6** was also kinetically competitive with DKA **1**. Deprotection of the benzyl ether moiety in **6** by hydrogenation led to **7** having the desired phenolic function in the meta position. **7** was even more potent than the previous compound, showing an $IC_{50} = 5.8$ μM (Mg^{2+}) and submicromolar activity in the presence of Mn^{2+}.

The moieties essential for chelation were investigated to understand the minimal requirement for the activity of this new class of HCV NS5b polymerase inhibitors. *O*-Methylation of the oxygen in the 4 and 5 positions of the pyrimidine core (Table 1, compounds **8** and **9**) led to inactive compounds, even in the presence of Mn^{2+}, indicating that the chelating properties were completely abolished. To complete the definition of the pharmacophore of the chelating moiety, **8** was decarboxylated to give **10**, which was also inactive even in the presence of Mn^{2+}. Thus, the carboxylic acid was the third essential moiety of the active site anchor (Table 1, compound **10**). All compounds were also tested in the HCV replicon assay, and they were neither active nor toxic up to 50 μM. The log D of compounds **5** and **6** are -1.05 and -0.22, respectively: this property could influence the cell permeability of these molecules in the cell-based assay. In addition, the lack of activity in this system is most likely due to the intrinsically modest enzyme inhibition.

The dihydroxypyrimidine carboxylic acid has several possible tautomeric forms in solution. The ^1H NMR spectra revealed that in DMSO the compounds are present mainly in one form (dihydroxy pyrimidine), but this does not exclude the possibility that upon binding with Mg^{2+} or Mn^{2+} the equilibrium shifts toward a pyrimidinone form (Figure 4, **3B**) and that this is the active tautomer.

To establish if the hydroxypyrimidinone **3B** is able to chelate the Mg^{2+} in the active site, the N-3 methylpyrimidinone of the most active compound **7** was prepared giving **11** that showed $IC_{50} = 6.0$ μM (Mg^{2+}) and $IC_{50} = 1.0$ μM (Mn^{2+}) (Figure 5).

The shift in potency in the presence of the two metals indicated that the *N*-methylpyrimidinone has the same behavior of DKA **1**, meconic acid derivative **2**, and dihydroxypyrimidine **3**, suggesting that all four classes share the same mechanism of action. What remains

Table 2.

compd	HIV RT IC$_{50}$/μM^a	HIV Int IC$_{50}$/μM^b	Klenow IC$_{50}$/μM^a
3	>100	>50	>100
5	23	>50	>100
6	>100	>50	>100
7	>100	>50	>100

^{a} Polymerase assay: see Supporting Information. ^{b} See ref 22.

unproven is if the dihydroxypyrimidine is present in the pyrimidinone form **3B** on binding with the cations Mg^{2+} or Mn^{2+} in the enzyme active site.

Active compounds were also tested against HIV-RT, HIV-integrase, and Klenow DNA-dependent DNA polymerase to measure their selectivity (Table 2). The original lead **5** was unselective, having similar activity on HIV-RT and HCV NS5b Pol. This undesirable property disappeared with all the other compounds. While aryl-DKAs have been reported as potent HIV integrase inhibitors,[22] our compounds showed no detectable inhibition against this target at tested concentration. These very preliminary results show that the substitution of aryl group in this new class of inhibitors can modulate the selectivity and potency. These results are in agreement with the function of the aryl moiety in DKA series, where it performs the same role.

It was then established whether the new class of inhibitors was more chemically or biologically stable than DKA's and meconic acid derivatives. Both **3** and **5** were completely stable in 1 N HCl solution and in aqueous media at pH = 7.4 after 24 h at room temperature; in contrast, **1** and **2** were almost completely decarboxylated in acid media. The stability of **5** and **7** were also measured in the presence of glutathione. On incubation for 2 h with and without GSH, in the same buffer and temperature conditions, the compounds proved to be completely stable. In contrast the DKA **1** was more than 50% consumed in the same experiment. Finally ^{3}[H]-**3** was used to measure the irreversible covalent binding of this inhibitor to rat liver microsomes in the presence of NADPH. In both cases, after 4 h of incubation, the residual radioactivity was less than 2 pmol/mg protein, indicating that compound **3**, the prototype of the series, does not bind irreversibly to rat liver microsome protein. In contrast the DKA showed a high level of irreversible binding (>400 pmol/mg protein).

Scheme 1 depicts a general procedure for the synthesis of dihydroxypyrimidines.[23] The appropriately substituted benzamide oxime was reacted with dimethyl acetylendicarboxylate to afford the Michael adduct as a mixture of cis−trans isomers which, on heating in refluxing xylene, probably underwent a Claisen rearrangement and cyclization to afford the methyl-5,6-dihydroxy-2-arylpyrimidine-4-carboxylate. This was hydrolyzed to the carboxylic acid **3** (R = H) or **6** (R = 3-OBn) by heating with an excess of NaOH. Hydrogenation of the benzyl ether of **6** gave **7**, which when heated with 6 N HCl afforded the 2-aryl-4,5-pyrimidinediol **10**.

The synthesis of the methylated derivatives **8** and **11** started with the 3-p-methoxybenzyl-protected dihydroxypyrimidine **14** (Scheme 2), which was prepared according to the above procedure.

After benzoylation of the 5-hydroxy group, reaction with methyl iodide and cesium carbonate in THF afforded a mixture of the methylated derivatives **15** and

Scheme 1. Synthesis of Compounds **3**, **6**, **7**, **10**^{a}

12 (R=H)
13 (R=3-OBn)

3 (R=H)
6 (R=OBn)

7 (R=OH)

10 (R=OH)
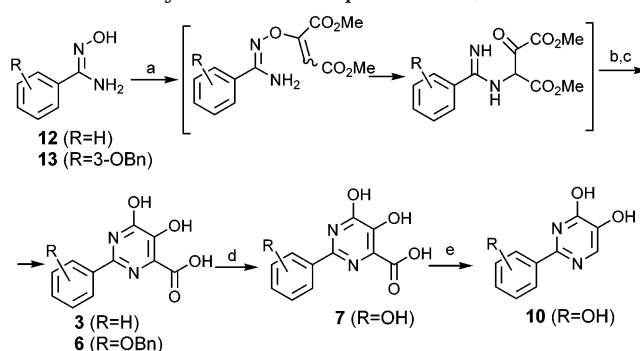
^{a} Reagents and conditions: (a) DMAD (1.2 equiv), CHCl$_3$ or MeOH, 50 °C, 2 h; (b) xylene, 140 °C, 12−18 h; (c) 2N NaOH (3 equiv), MeOH, 50 °C; (d) Pd/C, MeOH, H$_2$, rt; (e) 6 N HCl, reflux, 30 min.

Scheme 2. Synthesis of Compounds **8**, **9**, and **11**^{a}

4 (R=4-Cl)
14 (R=3-OPMB)

15

16

9

8

11
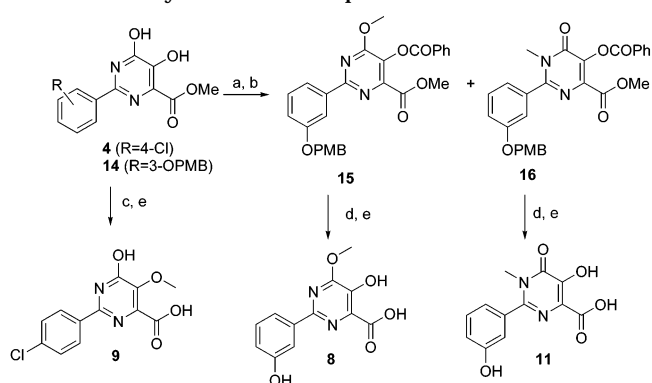
^{a} Reagents and conditions: (a) (PhCO)$_2$O (1.2 equiv), Pyr (3 equiv), CH$_2$Cl$_2$; (b) CH$_3$I (3 equiv), CsCO$_3$ (2 equiv), THF, 40 °C, 5 h; (c) 2M TMSCHN$_2$ soln in hexane (1.1 equiv × 5), MeOH, 4 °C, 12 h; (d) CH$_2$Cl$_2$: TFA (8:2 v/v), 30 min; (e) 2 N NaOH (3 equiv), MeOH, 50 °C.

16 (60−70% yield, 40:60 ratio), which were purified by flash chromatography. The p-methoxybenzyl group was cleaved by trifluorocetic acid in dichloromethane, and subsequent basic hydrolysis afforded **8** and **11**. The reaction of **4** with TMS-diazomethane in methanol followed by basic hydrolysis afforded the 5-O-methyl derivative **9**.

All compounds were fully characterized by ^{1}H NMR and LC-MS and HRMS. For unambiguous structural elucidation of compound **8**, **9**, and **11**, HMBC NMR was performed.

Two novel classes of HCV NS5b polymerase inhibitors were discovered: 4,5-dihydroxypyrimidine-6-carboxylic acid derivative **3** was designed from the other two leads, DKA **1** and meconic acid derivative **2**, originally discovered by screening. The corresponding N^3-methylpyrimidinone was synthesized in order to investigate the importance of keto−enol tautomerism of the 5-hydroxy group. The compounds were also tested against other RNA and DNA polymerases and HIV integrase proving that it is possible to achieve selectivity. Essential structural features of these new classes of HCV polymerase inhibitors necessary for activity were identified; the mechanism of action is similar to the other two leads from screening. The novel series offers considerable improvements over the original leads in druglike characteristics, being stable in acid and in the presence of GSH, and not covalently binding to rat liver microsomes.

Supporting Information Available: General synthetic procedures, ^{1}H NMR and HRMS data, and biological evaluation for compounds listed in all tables. This material is available free of charge via Internet at http://pubs.acs.org.

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