Discovery of α,γ-Diketo Acids as Potent Selective and Reversible Inhibitors of Hepatitis C Virus NS5b RNA-Dependent RNA Polymerase

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Abstract: α,γ-Diketo acids (DKA) were discovered from screening as selective and reversible inhibitors of hepatitis C virus NS5b RNA-dependent RNA polymerase. The diketo acid moiety proved essential for activity, while substitution on the γ position was necessary for selectivity and potency. Optimization led to the identification of a DKA inhibitor of NS5b polymerase with IC_{50} = 45 nM, one of the most potent HCV NS5b polymerase inhibitors reported.

Introduction. Hepatitis C virus (HCV) is a member of the flaviviridae viruses and is now recognized as the major cause of both parenterally transmitted and community-acquired non-A, non-B hepatitis (NANB-H). It was identified at the molecular level at the end of 1980s.[1] Although the number of new infections has been significantly reduced by the introduction of reliable blood testing, it has been estimated that at least 1% of the world population is affected by the disease. More than half of the affected individuals become chronically infected, and liver cirrhosis develops in at least 20% of this group. In addition, increased incidence of hepatocellular carcinoma in NANB-H patients suggests that HCV also plays a role in the process of hepatocarcinogenesis.

There is as yet no effective therapy for HCV-associated chronic hepatitis. Treatments with interferon α (IFN-α) alone or in combination with ribavarin are effective in controlling the disease only in a fraction of the patients. A high proportion of the infected individuals fail to respond to IFN-α therapy, and relapse after cessation of the treatment is not uncommon. There is thus an obvious need to develop effective therapeutic strategies to cure HCV-associated hepatitis.[2,3]

The most studied targets for anti-HCV therapy are the NS3 protease and the NS5b polymerase.[4] Several classes of NS3 protease inhibitors have been reported by us[5−8] and others,[9−12] mostly with a peptidic character. In the case of HCV NS5b polymerase, both nucleoside[13] and non-nucleoside inhibitors have appeared recently in the literature. The latter class contains different scaffolds that interact in allosteric binding sites of the protein,[14] and for some of them X-ray structures of enzyme−inhibitors complexes are also available.[15]

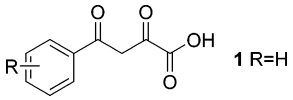


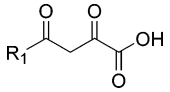
Figure 1. DKA lead **1** (depicted in the keto form) for HCV NS5b polymerase.

Random screening of more than 200 000 samples identified the α,γ-diketo acid **1** (Figure 1) as a low micromolar, specific, and reversible inhibitor of HCV NS5b polymerase with IC_{50} = 5.7 μM. At 500 μM, it did not inhibit the Klenow DNA polymerase and poliovirus RNA-dependent RNA polymerase. A weak inhibitory effect was observed on HIV-RT (IC_{50} = 54 μM). Recently, we have reported that the diketo acid (DKA) mechanism of inhibition is distinct from all the other known inhibitors of HCV polymerase reported so far in the literature; they are capable of interacting directly with metal ions present in the enzyme active site of HCV NS5b polymerase. They can be considered as product-like inhibitors showing a mechanism of action similar to that of foscarnet, a known pyrophosphate analogue.[4]

DKAs have been also reported as potent HIV integrase[16,17] and RNAse-H[18] inhibitors with HIV antiviral activity in cell-based assays. Similar inhibitors of influenza viruses are also known.[19]

Results and Discussion. Initial SAR showed that the aryl ring is absolutely required for activity. Its replacement with aliphatic groups such as methyl, t-Bu, and benzydryl group leads to compounds displaying IC_{50} > 50 μM (Table 1, **2**−**4**). Phenyl can be replaced with thiophene to yield **5** showing activity similar to that of the original lead **1**. However, more polar heterocycles such as 3- or 4-pyridine as well as 2-furan were not tolerated (Table 1, **6**−**8**).

Table 1. Replacement of Phenyl Moiety of DKA Lead



compd	R_1	IC_{50} (μM)[a]
1	Ph	5.7 ± 0.2
2	Me	>50
3	t-Bu	>50
4	$(Ph)_2CH$	>50
5	3-thiophene	4.5 ± 0.2
6	3-pyridine	>50
7	4-pyridine	>50
8	2-furan	>50

[a] For polymerase assay, see Supporting Information. Data are the mean of two to four independent experiments.

The DKA moiety was also explored, and it proved essential for the inhibitory activity. Simple modification led to the complete loss of inhibitory activity against the HCV NS5b polymerase (Table 2, **9**−**11**). Structure **1** is therefore the minimal scaffold capable of inhibiting the HCV NS5b polymerase. By use of this compound as a template, substitutions on the phenyl ring were introduced singularly or in tandem to explore structure−activity relationship.

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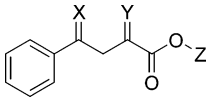
Table 2. DKA Acid Moiety Essential for Activity



compd	X	Y	Z	IC_{50} (μM)[a]
1	O	O	H	5.7 ± 0.2
9	O	O	Me	>50
10	O	OH	H	>50
11	H_2	O	H	>50

[a] For polymerase assay, see Supporting Information. Data are the mean of two to four independent experiments.

Initial studies on the para position revealed that only small substituents were tolerated, such as methyl **12** that retains the same potency, while the introduction of an ethyl **13** or t-Bu **14** resulted in a considerable loss of activity (Table 3).

Table 3. Para-Substituted DKA (Figure 1)

compd	R	IC_{50} (μM)[a]
1	H	5.7 ± 0.2
12	4-Me	5.0 ± 1.0
13	4-Et	47.0 ± 3.0
14	4-t-Bu	>50

[a] For polymerase assay, see Supporting Information. Data are the mean of two to four independent experiments.

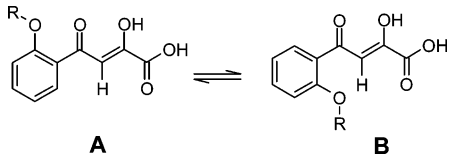
In contrast, substituents in the ortho position showed a significant improvement of activity with alkyl ethers, which were found to be the optimum functional group. Simple aliphatic chain ethers with a length of three carbon atoms (**15**) were preferred, and the potency dropped upon increasing chain length (**16**, **17**). Side chains containing aromatic moieties were not tolerated (**18**). Propyl side chains having a variety of terminal functional groups were explored and are allowed without a considerable improvement of activity against the NS5b polymerase (data not shown). The only exception was the nitrile **19**, which resulted in a 20-fold improvement of inhibitory activity with respect to the original screening lead, displaying IC_{50} = 350 nM (Table 4).

Table 4. Ortho-Substituted DKA (Figure 1)

compd	R	IC_{50} (μM)[a]
1	H	5.7 ± 0.2
15	2-O-propyl	3.9 ± 0.5
16	2-O-butyl	10.0 ± 1.0
17	2-O-pentyl	25.0 ± 2.0
18	2-O-Bn	44.0 ± 3.0
19	2-$OCH_2CH_2CH_2CN$	0.35 ± 0.04

[a] For polymerase assay, see Supporting Information. Data are the mean of two to four independent experiments.

Ortho ethers may adopt two different conformations. In one (Figure 2, **A**), the ortho aryl oxygen is close to the carbonyl oxygen of the DKA. In the second conformation (**B**), the ortho aryl oxygen is oriented toward the C3−H. This relative orientation (**B**) was consistent with results from NMR experiments probably because of electrostatically unfavorable interactions between the ether and C4 oxygen atoms in conformation **A**. NOE experiments indicated a distance larger than 4 Å between the proton in the ortho position of the phenyl ring and the vinylic proton C3−H of the enolic form of the DKA.



A **B**

Figure 2. Ortho ether substituted DKA **A** in a self-assembled conformation **B**.

The meta position of the phenyl ring appears to be more tolerant of size and nature of the substituents compared to the ortho and para positions. Simple *O*-alkyl side chains did not have the same effect obtained in the ortho position. Aromatic moieties were preferred, and different functional groups were tolerated. In the first selection of substituents, the choice was made to obtain similar size and shape (Table 5); benzoic amide **20**, phenyl sulfonamide **21**, benzylamine **22**, and benzyl ether **23** were compared. The resulting activities were all in the same range with the exception of **23**, which showed single-digit micromolar activity (IC_{50} = 8 μM). **24**, which had an inverse benzyl ether function with respect to **23**, showed a reduction in potency.

Table 5. Meta-Substituted DKA (Figure 1)

compd	R	IC_{50} (μM)[a]
1	H	5.7 ± 0.2
20	3-NHCOPh	11.0 ± 1.0
21	3-$NHSO_2$Ph	16.0 ± 1.5
22	3-NH-Bn	16.7 ± 1.5
23	3-O-Bn	8.0 ± 0.9
24	3-CH_2OPh	30 ± 4.0

[a] For polymerase assay, see Supporting Information. Data are the mean of two to four independent experiments.

On the basis of these results, the benzyl ether was chosen as a fixed fragment for further optimization. We explored the possibility of exploiting the same interactions in the meta-substituted series which render the *o*-3-cyanopropoxy substituted DKA **19** such a potent inhibitor. Molecular modeling indicated that the phenyl ring of the benzyl moiety prefers a coplanar arrangement relative to the DKA phenyl ring (Figure 3).

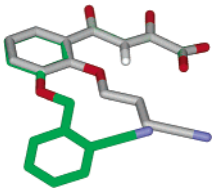


Figure 3. Superimposition of DKA **19** (gray) and **25** (green). Of four almost isoenergetic conformations of **25**, only the one most similar to **19** is shown.

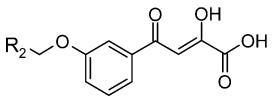
Introduction of a cyano group in position 2 of the benzyl moiety should place the cyano group in a position similar to that of **19** (Figure 3). Indeed, the optimal substituent was the 2-cyano benzyl ether **25** showing IC_{50} = 230 nM, 1.5-fold more potent than **19**.

The isomeric 3-cyano benzyl ether **26** was 15-fold less active with respect to **25**. **27** with a simple, more flexible aliphatic side chain having the same length from the oxygen of the ether to the nitrile proved 11-fold less active.

The nitrile group was left constant at the 2 position of the benzyl ether, and a variety of substituents were

added to this moiety to improve the potency (Table 6). Introducing a single bromine in the 5-position gave a 2-fold improvement in potency **28** (IC_{50} = 100 nM), while 3-chloro **29** was 1.5-fold more active (IC_{50} = 110 nM). The combination of two halogens generated **30**, which showed IC_{50} = 45 nM against the NS5b polymerase. **30** is one of the most potent inhibitors of HCV NS5b polymerase reported in the literature. **30** was highly selective with regard to the α,β and γ human DNA polymerases and HIV RT, showing IC_{50} > 5.0 μM and IC_{50} > 50.0 μM, respectively.

Table 6. Meta-Substituted Benzyl Ether



compd	R_2	IC_{50} (μM)[a]
25	2-CNPh	0.23 ± 0.02
26	3-CNPh	6.70 ± 0.8
27	CH_2CH_2CN	2.50 ± 0.5
28	2-CN-5-BrPh	0.100 ± 0.005
29	2-CN-3-ClPh	0.110 ± 0.010
30	2-CN-3,5-Cl_2Ph	0.045 ± 0.008

[a] For polymerase assay, see Supporting Information. Data are the mean of two to four independent experiments.

Meta ethers do not have the same self-assembled conformation as seen with the ortho ether, and consequently, the side chain could be located in either region of space with respect to the DKA moiety. To exclude the possibility that the side chains of **19** and **25** explore different regions of space generating different contacts with NS5b polymerase, a 2,5 disubstituted compound, **31** (Figure 4), was prepared bearing the best substitution found in both positions. It was essentially inactive (IC_{50} > 50 μM), suggesting that the two side chains occupy the same region as in Figure 3.

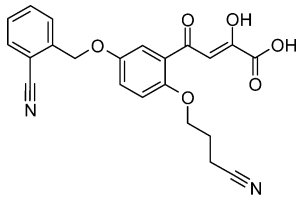
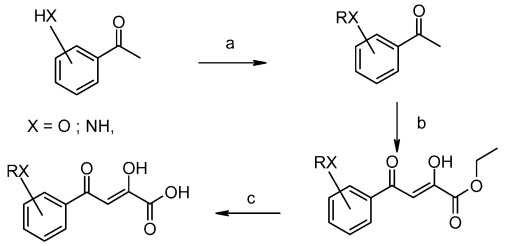


Figure 4. Disubstituted DKA **31**.

Synthesis. Scheme 1 depicts a general procedure for the synthesis of DKA. The appropriate substituted acetophenone is reacted with diethyl oxalate in the presence of sodium ethoxide in THF, and subsequent hydrolysis of the ethyl ester with sodium hydroxide in methanol gives the desired DKA. The two steps can be carried out separately or in one pot.

O-Alkylacetophenone precursors of **15**−**19**, **23**, and **25**−**30** were prepared by alkylation of the corresponding substituted hydroxyacetophenones with the appropriate bromoalkyl derivative in DMF in the presence of Cs_2CO_3 at 80 °C for 12 h (Scheme 1, step a). For **31**, 2,5-dihydroxyacetophenone was used as the starting material and the 5′-hydroxy functional group was selectively alkylated by reaction with equimolar 2-cyanobenzylbromide in the presence of Cs_2CO_3 in DMF at 60 °C. Further alkylation of the 2-hydroxy group with bromovaleronitrile afforded the required precursor of **31**. Benzoylation, sulfonylation, or alkylation of 2-aminoacetophenone generated the suitable acetophenone precursors of **20**−**22**. Displacement with phenol of the 3-bromotoluolacetophenone gave the precursor for **24**. Bromination of suitably substituted toluene derivatives afforded the bromo derivative used for the alkylation of 3-hydroxyacetophenone precursors of **25**−**26** and **28**−**30**. All compounds were fully characterized by ^{1}H NMR, LC−MS, and HRMS.

Scheme 1. Synthesis of **1**−**8** and **12**−**30**[a]



[a] Reagents and conditions: (a) Br−R or Cl−R, Cs_2CO_3 DMF, 80 °C, 12 h for X = O, NH; RCOCl, THF, $N(Et)_3$, room temp for X = NH; RSO_2Cl, Py, room temp, 8 h for X = NH; (b) diethyl oxalate, NaOEt, THF, room temp; (c) NaOH in MeOH.

Conclusion. By screening, we have discovered a novel class of HCV NS5b polymerase inhibitors selective against other RNA and DNA polymerases. Essential structural features of DKAs necessary for activity were identified, and the lead **1** that showed low micromolar activity was converted into **30** with IC_{50} = 45 nM, one of the most potent HCV NS5b polymerase inhibitors reported in the literature.

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

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