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Dual inhibition of HCV and HIV by ring-expanded nucleosides containing the 5:7-fused imidazo[4,5-*e*][1,3]diazepine ring System. In vitro results and implications

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Abstract

Examples of ring-expanded nucleosides (RENs), represented by general structures **1** and **2**, exhibited dual anti-HCV and anti-HIV activities in both cell culture systems and the respective target enzyme assays, including HCV NTPase/helicase and human RNA helicase DDX3. Since HCV is a leading co-infection in late stage HIV AIDS patients, often leading to liver cirrhosis and death, the observed dual inhibition of HCV and HIV by the target nucleoside analogues has potentially beneficial implications in treating HIV patients infected with HCV.

Keywords

Ring-expanded nucleosides; imidazo[4,5-*e*][1,3]diazepines; organic synthesis; antiviral compounds; *in vitro* screening; hepatitis C virus (HCV); human immunodeficiency virus (HIV); dual inhibitors of HCV and HIV; inhibition of HCV NTPase/helicase; inhibition of RNA helicase DDX3

Highly active antiretroviral therapy (HAART), employing a three-drug regimen acting on different stages of the viral life cycle, has dramatically increased the survival rate of the HIV-infected individuals, and has transformed Acquired Immunodeficiency Syndrome (AIDS) into a controllable chronic illness.^{1, 2} A fateful outcome of the chronic HIV

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Supplementary Data

Supplementary data associated with this article, including organic synthetic procedures, spectral and microanalytical data as well as experimental procedures used for biochemical and biological assays, can be found in the online version of the journal at <http://dx.doi.org/10.1016/j.bmcl.2013.12.121>. These data include MOL files and InChIKeys of the most important compounds described in this article.

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condition, however, is the progressively weakening immune system since HIV primarily infects the CD4 lymphocytes which help the body fight infections.^{3–5} This makes HIV patients vulnerable to opportunistic co-infections, including but not limited to that caused by the Hepatitis C virus (HCV).^{6–9} The end-stage liver diseases caused by hepatitis viral infection is now one of the major causes of death (>50%) in HIV patients in the Western World.^{10–12} In a recent study exploring the cause of death in HIV patients, a vast majority of the dead had tested positive for antibodies to HCV.¹³ Out of the HIV opportunistic infections, HCV in particular has lately taken the center stage, causing alarms in the AIDS research community for many reasons, including (a) the vastly successful HAART therapy is considerably less effective with HIV patients co-infected with HCV,¹⁴ (b) the protease inhibitors used in the HAART therapy exert a significant degree of extra strain on the liver that is already stressed by HCV.^{14, 15} This results in dramatic exacerbation of HCV and its accelerated progress to liver cirrhosis and death. Thus, patients on HAART therapy are even at more risk for liver diseases,^{14, 15} (c) the approved anti-HCV therapy with a combination of α -interferon and ribavirin was shown to decrease the potency of anti-HIV therapy because of the suspected molecular interaction of ribavirin with the reverse transcriptase inhibitors used in HAART, resulting in the latter's diminished effectiveness.¹⁶ It is also not yet clear how the recently approved protease inhibitors for HCV treatment, including Victrelis (boceprevir),¹⁷ Incivek (telaprevir),¹⁷ and Olysio (simeprevir)¹⁷ would affect disease progression of HIV patients infected with HCV. For these reasons, mutually compatible anti-HCV and anti-HIV drugs are needed to combat HCV co-infection in HIV patients. These drugs should neither exacerbate the clinical manifestations of the co-infection nor diminish the efficacy or effectiveness of the therapy used for treatment of the individual infection.

We have recently reported¹⁸ the *in vitro* anti-HIV activity of a series of ring-expanded nucleosides (RENs) containing imidazo[4,5-*e*][1,3]diazepine ring system, represented by general structural formulas **1** and **2**. Out of the many compounds screened for inhibition of HIV-1 replication in virus-infected T cell line (MT4 cells), compounds **1f** and **2e** were identified as the lead compounds with low micromolar IC₅₀'s. Both compounds contained the long (18-carbon) alkyl chain at the C-6 position of the heterocycle, and differed from one another in their stereochemical configuration at the anomeric junction and the lack of 2'-OH group in **2e**. Neither compound was significantly toxic in *ex vivo* cell culture or *in vivo* in mice.¹⁸ Both compounds strongly inhibited cellular RNA helicase DDX3, which the virus is believed to exploit for its replication as it lacked its own helicase.¹⁹ Helicases are capable of unwinding duplex RNA and DNA structures by disrupting the hydrogen bonds that keep the two strands together.^{20, 21} This unwinding activity, which is normally accompanied by simultaneous hydrolysis of an NTP (ATP or GTP),²² is essential for the virus replication.

Several years ago, we had also reported²³ that a wide variety of RENs, containing both the imidazo[4,5-*e*][1,3]diazepine and imidazo[4,5-*e*][1,2,4]triazepine ring systems, potently inhibited the virus-encoded NTPases/helicases of several RNA viruses belonging to the *Flaviviridae* family, including but not limited to the West Nile Virus (WNV), Hepatitis C Virus (HCV), and the Japanese Encephalitis Virus (JEV). Subsequently, we had discovered²⁴ that compounds represented by general structural formulas **1** and **2** were especially effective in inhibition of the WNV NTPase/helicase. To our surprise and delight, these RENs failed to inhibit a truncated form of the human helicase Suv3₍₀₁₋₁₅₉₎, which we had included in the study in order to assess their selectivity and toxicity.²⁴ Because of the perceived serious threat of WNV epidemic in North America in the early 2000's, our research efforts during that time, along with many other laboratories in US and Canada, were largely focused on WNV. In light of the promising results of **1** and **2** with HIV inhibition, coupled with the increasing problem of HCV co-infection in HIV patients as described above, and not to mention that HCV belongs to the same viral family as WNV,

against which **1** and **2** had already exhibited potent antiviral activity, it was only logical to extend our investigation of these RENs to HCV, as elaborated in this paper.

Syntheses of a majority of the compounds necessary for our present study, listed under general structural formulas **1** and **2**, were carried out by us earlier for the mentioned HIV and WNV studies^{18, 24} and so will not be repeated here. Synthesis of the two new compounds used in this study, **2a** and **2b**, is outlined in Scheme 1. Butyl imidazole-4,5-dicarboxylate (**3**) was glycosylated with 2-deoxy-3,5-di-*O*-*p*-toluoyl- β -D-erythropentofuranosyl chloride (**4**),²⁴ generated in situ from the corresponding α sugar, in the presence of *N,N,N',N'*-tetramethyl-1,8-naphthalenediamine (proton sponge) to obtain butyl 1-(2'-deoxy-3',5'-di-*O*-*p*-toluoyl- α -D-erythropentofuranosyl)-4,5-imidazolidine-carboxylate (**5**) in 62% yield. Condensation of the latter with (*N*-octyl)- and (*N*-decyl)guanidinium nitrates, freshly generated from the reaction of 3,5-dimethylpyrazole-1-carboxamide nitrate with *N*-octyl- and *N*-decylamine, respectively,²⁴ catalyzed by sodium methoxide in methanol, afforded **2a** and **2b** in 92 and 94%, respectively. Both compounds were fully characterized by spectroscopic and microanalytical data.²⁵ The NTPase/helicase of HCV was expressed in *E. coli* and purified by affinity chromatography.²⁵ The homogeneity of the enzyme preparation was verified by Coomassie Blue staining. In order to monitor the inhibitory potential of RENs toward helicase activity of HCV NTPase/helicase, radiolabelled partially double-stranded (ds) DNA was used as substrate.²⁵ Out of the 11 compounds listed under **1** and **2** that were biochemically screened, only **1f** and **2e** showed promising anti-helicase activity of HCV NTPase/helicase as shown in Table 1. This is remarkably consistent with what we had earlier observed with anti-HIV activity.¹⁸ A long alkyl chain of a minimum of 18 carbon atoms, attached at the 6-position of the heterocycle, appears to be necessary for both anti-HIV and anti-HCV activities. We then repeated our above experiment of HCV NTPase/helicase with **1f** and **2e** using a double-stranded RNA substrate, and found that the compounds were equally effective as with a DNA substrate within a margin of experimental error. All other compounds listed under **1** and **2** failed to show activity even up to 300 $\mu\text{g/mL}$, although some of them (e.g. **1c** and **1d**) had earlier exhibited excellent activity against WNV NTPase/helicase.²⁴

In light of the above encouraging anti-enzymic activity, both **1f** and **2e** were further screened for *in vitro* anti-viral activity against the whole virus as well as cellular toxicity in a Primary Replicon assay.^{26, 27} The results collected in Table 1 reveal that both activity and toxicity profiles of **1f** and **2e** are closely comparable to that of interferon- α , one of the reference compounds normally used for the assay. However, the two compounds showed significantly higher antiviral activity and much lower toxicity as compared with those of ribavirin, the second reference compound used for the assay. A combination therapy with IFN- α and ribavirin has long been the FDA-approved treatment for HCV.^{28, 29}

Since the viral NTPase activity is often coupled to its helicase activity as mentioned earlier,²² an important question to address was whether compounds **1f** and **2e** also inhibited the NTPase activity of HCV NTPase/helicase. This would give further insight into mechanism of activity since the compounds could simply be binding to the NTP binding site of HCV NTPase/helicase to cause interference in NTP hydrolysis that is required for the viral helicase activity. The HCV NTPase activity was assessed²⁵ by a standard assay that determines the amount of P_i released from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ during the enzyme-mediated hydrolysis. The ATPase activity was measured as a function of increasing concentrations of the inhibitors. However, when investigated at ATP concentration equal to the K_M value of the enzyme (105 μM for HCV NTPase/helicase),²³ neither of the two compounds inhibited the hydrolytic activity of the enzyme even up to the concentration of 0.7 mM. The successive lowering of ATP concentration down to value corresponding to 1×10^{-5} of K_M also did not lead to any observable inhibition of the ATPase activity. These results suggest that these inhibitors did not act *via* the ATP (NTP)-binding site of HCV NTPase/helicase as ATP-competitive inhibitors.

As anti-HCV activity was detected in the primary replicon assay as mentioned above, more thorough investigations were then made on promising RENs **1f** and **2e** in order to determine their IC_{50} values (see Figure 1). The BM4,5 cell line (a human hepatoma Huh7 cell line harboring an HCV genotype 1b subgenomic replicon) was used, which is a relevant model for studying the effect and mechanism of action of antiviral agents. Dose response curves were generated and IC_{50} values were computed. Compounds **1f** and **2e** were found to have IC_{50} values of $\sim 16 \mu\text{M}$ and $18 \mu\text{M}$, respectively. They were also found to exhibit low toxicity, if any, at the concentration levels tested, employing Neutral Red Dye Assay^{30, 31} (Figure 2).

Finally, in order to verify if the observed activities of **1f** and **2e** are not solely the result of simple, long-chain alkylamines present at their 6-position, we separately tested two long-chain alkylamines, $\text{CH}_3(\text{CH}_2)_{17}\text{NH}_2$ and $\text{CH}_3(\text{CH}_2)_{13}\text{NH}_2$ for their anti-HCV and anti-HIV activities *in vitro*, and found them totally inactive against both viruses. This suggests that the entire structural framework of **1f** and **2e**, and not simply the long alkyl chains attached at their 6-position, is necessary for the observed antiviral activities.

In conclusion, we have discovered two nucleoside analogues **1f** and **2e** that show promising *in vitro* antiviral activity against both HCV and HIV. The long-chain alkyl moiety of a minimum of 18-carbon length attached to the 6-position of each is a requirement for activity. At this time, it is not known if a longer alkyl chain would further enhance each activity or what the threshold of chain length is for optimum antiviral activity. In light of HCV being a major co-infection in HIV patients in the Western World, RENs might represent a new concept in the treatment of both viruses, and merit further exploration. It should also be noted that while vast majority of current HCV research is targeted to the NS5 region of HCV encoding its polymerase RdRp (RNA-dependent RNA polymerase), our target is the relatively little explored NS3 region of HCV that encodes its NTPase/helicase. Nonetheless, a distinct possibility that compounds **1f** and **2e** could also undergo viral and/or host kinase-

mediated *in vivo* phosphorylation to their corresponding 5'-triphosphates, and the subsequent RdRp-catalyzed incorporation into viral genome, followed by chain-termination, cannot be ruled out at this point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Anti-HCV activity of **1f** and **2e** was assayed in BM 4,5 cell line carrying subgenomic HCV replicon in HuH7 human hepatoma cells described by Guo *et al.* (2001).^{25, 32}

Fig. 2.

The cytotoxicity was determined using the Neutral Red Dye assay.^{30, 31}

Scheme 1.

Table 1

Inhibitory effect of RENs **1f** and **2e** against HCV NTPase/helicase[∞] as well as against the whole virus in tissue culture system assessed by Primary Replicon Assay^ψ

Compound No.	Inhibition of Helicase Activity [∞] of HCV NTPase/Helicase IC ₅₀ (μg/mL) ^a	Antiviral Activity ^ψ HCV RNA ^b % Control	Toxicity β-Actin RNA ^c %Control	SI ^d Toxicity/Antiviral Activity
1f	6±0.1	9±13	98±3	10.89
2e	5.5±0.2	10±0.01	99±2	9.9
IFN-α (REF1) (10 IU/mL)	-	10±1	108±4	11.3
Ribavirin (REF2)	-	89±10	12±1	0.14

[∞]The inhibitory potential of the compounds was expressed as the inhibitor concentration at which 50% of the unwinding activity was measured. The helicase activity of the enzyme measured in the absence of the compounds was referred to as 100%.

^ψThe antiviral activity is based on a primary replicon assay^{26, 27} in Huh7 ET cell line.

^aThe term IC₅₀ is defined as the concentration of REN required for 50% inhibition of enzyme activity.

^bHCV RNA-derived LUC activity is used as an indirect measure of HCV RNA levels.

^cβ-Actin RNA level is used as a positive control for cellular RNA in order to compute cytotoxicity.

^dSelectivity index (SI) is represented as a ratio of the levels of β-Actin RNA/HCV RNA.