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Synthesis and in vitro anti-hepatitis B and C virus activities of ring-expanded ('fat') nucleobase analogues containing the imidazo[4,5-*e*][1,3]diazepine-4,8-dione ring system

Peng Zhang,[a] Ning Zhang,[a] Brent E. Korba[b] and Ramachandra S. Hosmane[a,*]

[a]*Laboratory for Drug Design and Synthesis, Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, 1000 Hilltop Circle, Baltimore, Maryland 21250, USA*
[b]*Division of Molecular Virology and Immunology, Georgetown University Medical Center, Rockville, MD 20850, USA*

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Abstract—As part of our structure–activity relationship studies, we report here the synthesis and in vitro anti-HBV and anti-HCV activities of a number of ring-expanded ('fat') nucleobases containing the imidazo[4,5-*e*][1,3]diazepine-4,8-dione ring system. One of the compounds, **ZP-88**, exhibited a good activity/toxicity profile against HBV by inhibition of the synthesis of extracellular virion release (EC_{50} = 1.7 μM, CC_{50} = 286 μM, SI = 168) and intracellular HBV replication intermediates (EC_{50} = 8.4 μM, CC_{50} = 286 μM, SI = 34) in cultured human hepatoblastoma 2.2.15 cells. By contrast, most of the compounds tested against HCV had only marginal activity/toxicity profile, although that was still better than that of the reference compound ribavirin.

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are two of the most dreadful viruses currently challenging the global human health.[1,2] HBV has infected over two billion people worldwide and 1.5 million in U.S., and the infection from HCV stands at >175 and 5 millions, respectively.[3–5] Although both cause chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma,[6,7] they belong to two totally different viral families. While HBV is a double-stranded DNA virus classified under *Hepadnaviridae*,[8,9] HCV has a single-stranded, positive-sense RNA genome and is classified under *Flaviviridae*.[10] A recombinant vaccine is available for treatment of human HBV infection, but no such vaccine exists as yet against HCV. Furthermore, while there are a few, effective, FDA-approved oraltherapies, such as Lamivudine (3TC)[11] and Adefovir Dipivoxil,[12] available for HBV infection, the clinical benefits of the existing treatment options against HCV infection are limited, and include a far less effective treatment with a combination therapy including interferon-α and a non-selective and/or toxic drug ribavirin.[13,14] Although several potent inhibitors of HCV replication have recently been reported,[15–18] their clinical efficacy and safety have yet to be proven. Both HBV and HCV transmission occurs primarily through blood and blood-related products,[19,20] and is most efficient via percutaneous mode (needles),[21] although HCV is also common in sexually promiscuous individuals.[22] In addition, both HBV and HCV are the major co-infections in HIV patients and are the frequent causes of the end-stage liver disease associated with death of HIV patients.[23] We report herein the latest results of our continued structure–activity relationship studies against HBV[24] and HCV[25] based on ring-expanded heterocycles and nucleosides, containing the title imidazo[4,5-*e*][1,3]diazepine-4,8-dione ring system.

A number of ring-expanded ('fat') heterocycles and nucleoside analogues containing the title ring system **I** have been synthesized in this laboratory in recent years and were biologically screened in vitro against both HBV[24] and HCV.[25] In the case of HBV, the compounds were specifically tested for inhibition of replication in cultured human hepatoblastoma 2.2.15 cells,[24] whereas screening against HCV involved the assessment of inhibition of the viral NTPase/helicase,[25] a crucial enzyme involved in replication. A number of compounds exhibited potent activities against both viruses with IC_{50} values in the micromolar range, and little or low toxicity to the host cells (HBV)[24] or the host enzyme (HCV).[25]

Keywords: HBV; HCV; Inhibition; Ring-expanded nucleobases; Imidazo[4,5-*e*][1,3]diazepines.
* Corresponding author. Tel.: +1 410 455 2520; fax: +1 410 455 1148; e-mail: hosmane@umbc.edu

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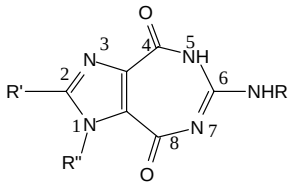
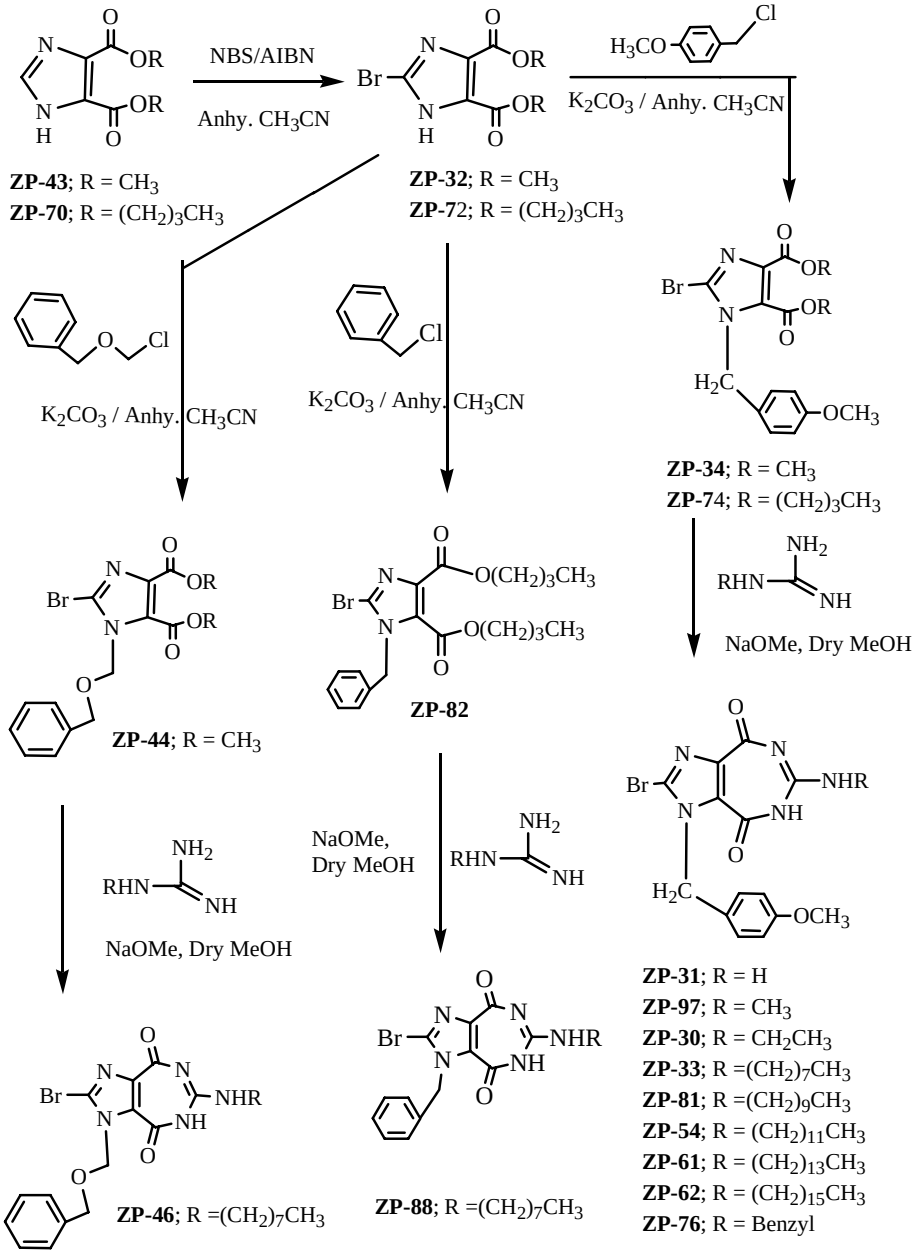


I

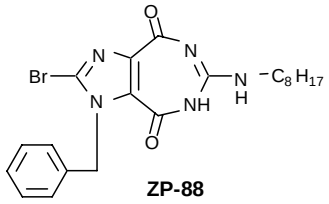
An interesting outcome of the above investigations was that a number of ring-expanded heterocyclic aglycons, in addition to the corresponding nucleoside analogues, were active against *Flaviviridae*.[25,26] This is what kindled our interest in the present work using ring-expanded heteocycles. We reasoned that the biological outcome with the aglycons alone is likely to shed more light on the mechanism of viral inhibition by ring-expanded molecules in general. To this end, we set out to synthesize and screen a number of judiciously chosen heterocycles bearing the structural skeleton **I**. In our previous studies, most of the compounds screened contained substitutions at positions 1 and 6 of the general structure **I**,[25,26] while position 2 remained largely unexplored. As it is not yet known if a hydrophobic or a hydrophilic substituent at position 2 would enhance the antiviral activity, we intuitively chose to put a bromo substituent at this position. This was mainly for two reasons: one, bromine atom is reasonably large and polarizable and thus can act as a quick, albeit approximate, dimensional probe of enzyme (or receptor) active site, and two, it can be easily manipulated into either hydrophobic or hydrophilic substituents for further structure–activity relationship (SAR)



Scheme 1.

studies. Therefore, keeping the 2-bromo group as a common functionality, we synthesized a series of compounds with structural variations at the 1- and 6-positions, as outlined in Scheme 1. The synthesis is straightforward and involved condensation of the appropriate 1-substituted-2-bromoimidazole-3,4-dicarboxylic acid esters with a variety of guanidine derivatives. The required 2-bromo derivatives of imidazole 4,5-diesters were prepared by bromination of the latter with *N*-bromosuccinimide (NBS) in the presence of a free radical initiator

Table 1. Anti-HBV activity of a ring-expanded heterocycle in vitro[a]



ZP-88

Compound ID	Antiviral activity EC_{50} (μM)[c]		Antiviral activity EC_{90}(μM)[c]		Toxicity CC_{50}[b] (μM)	Selectivity index (SI)[f] CC_{50}/EC_{50}	
	Virion[d]	HBV RI[e]	Virion[d]	HBV RI[e]		Virion[d]	HBV RI[e]
ZP-88	1.7 ± 0.2	8.4 ± 0.8	6.9 ± 0.7	28 ± 2.2	286 ± 20	168	34
3TC (Ref.)	0.048 ± 0.005	0.251 ± 0.029	0.151 ± 0.013	0.648 ± 0.062	2159 ± 78	44,979	8602

[a] Appropriate concentrations of the test compounds were added daily for nine days to the HBV producing 2.2.15 cells. Culture medium was collected daily and tested for extracellular (virion) HBV DNA at days 0, 3, 6, and 9. Cells were lysed 24 h after day 9 for the analysis of intracellular HBV replication intermediates (HBV RI). HBV virion DNA and intracellular HBV DNA RI levels in the cells were measured by blot hybridization methods (Southern and dot blot) and [P][32] labeled HBV-specific probes.

[b] CC_{50}, is the drug concentration at which a twofold reduction of neutral red dye uptake from the average value in the untreated cultures was observed.

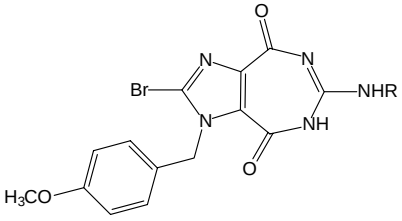
[c] $EC_{90}$and EC_{50} are concentrations which give 90% and 50% inhibition of viral replication in the cell cultures, respectively. Values presented (±standard deviation) were calculated by linear regression analysis using data combined from all treated cultures; standard deviations (SD) were calculated by using the standard error of regression generated from the linear regression analysis.

[d] Extracellular HBV virion DNA.

[e] Intracellular HBV DNA replicative intermediates.

[f] Selectivity index was calculated as CC_{50}/EC_{50} ratio.

Table 2. Anti-HCV activity of ring-expanded heterocycles in vitro[a]



Compound ID	No. of carbons present at position-6 *n*	Antiviral activity HCV RNA[b] % control	Toxicity β-actin RNA[c] % control	Selectivity index SI[d] toxicity/antiviral activity
ZP-31 (R = H)	0	62 ± 1	89 ± 4	1.435
ZP-97 (R = CH_3)	1	33 ± 3	48 ± 2	1.453
ZP-30 (R = C_2H_5)	2	34 ± 4	65 ± 9	1.912
ZP-33 (R = C_8H_{17})	8	32 ± 13	69 ± 3	2.155
ZP-81 (R = $C_{10}H_{21}$)	10	53 ± 6	32 ± 1	0.604
ZP-54 (R = $C_{12}H_{25}$)	12	47 ± 7	47 ± 3	1
ZP-61 (R = $C_{14}H_{29}$)	14	64 ± 4	34 ± 7	0.532
ZP-62 (R = $C_{16}H_{33}$)	16	70 ± 10	3 ± 1	0.043
ZP-76 (R = Benzyl)	Benzyl group	55 ± 1	69 ± 2	1.255
ZP-46	8	29 ± 7	61 ± 8	2.10
ZP-88	8	78 ± 5	83 ± 6	1.06
Interferon-α (10 IU/mL)	Ref. 1	10 ± 1	108 ± 4	11.3
Ribavirin	Ref. 2	89 ± 10	12 ± 1	0.42

[a] The antiviral activity is based on a primary assay employing 10 μM concentrations of the test compound for determination of both antiviral activity and toxicity. The assay was performed using an Huh7 ET cell line, which contains the HCV RNA replicon with a stable luciferase (LUC) reporter.

[b] HCV RNA-derived LUC activity is used as an indirect measure of HCV RNA levels.

[c] β-Actin RNA level is used as a positive control for cellular RNA in order to compute cytotoxicity.

[d] Selectivity index (SI) is represented as a ratio of the levels of β-actin RNA/HCV RNA.

azobisisobutyro-nitrile (AIBN), employing a literature procedure.[27] The necessary guanidine derivatives for condensation, when not commercially available, were synthesized by reaction of 3,5-dimethylpyrazole-1-carboxamidine nitrate with the appropriately substituted amine in methanol at reflux, using the procedure of Scott et al.[28] All new intermediates and final products were fully characterized by spectroscopic and microanalytical data.[29,30]

The target compounds were screened against HBV and HCV through contractual arrangements with the National Institute of Allergy and Infectious Diseases (NIAID), employing standard protocols, published on NIAID-AACF website.[31] Anti-HBV activity and toxicity against confluent 2.2.15 cells were determined by the published procedure of Korba and Gerin.[32] Anti-HCV activity and toxicity were assessed by the HCV RNA Replicon assay of Krieger et al.[33] The biological screening results for anti-HBV and anti-HCV activities of the target compounds are collected in Tables 1 and 2, respectively. Out of the several compounds tested, only **ZP-88** exhibited promising anti-HBV activity/toxicity profiles as shown. The compound was also found to be active against the 3TC-resistant mutant L180M (EC_{50} = 12 μM). On the other hand, all of the heterocycles tested had at least some activity against HCV.

As is evident from the data in Table 2, the best HCV activity/selectivity profile was obtained with an eight-carbon chain at position-6 (compound **ZP-33**). All compounds, but one (**ZP-62**), exhibited better toxicity/selectivity ratio (selectivity index) than the reference compound ribavirin that is currently being used in combination with interferon-α to treat HCV infection. Nevertheless, the observed activities are considerably lower than that of interferon-α, the other reference compound employed in the assay.

In conclusion, the heterocyclic aglycons of ring-expanded nucleosides exhibit in vitro activity against both hepatitis viruses, HBV and HCV, despite the fact that the two viruses have distinctly different genomes. Further structure–activity relationship (SAR) and mechanistic studies are warranted for enhancing the toxicity/activity profile of these compounds, and such an endeavor is currently in progress

Acknowledgments

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Supplementary data

Experimental procedures as well as physical, spectral and analytical data (six pages) for the key compounds reported in this article can be found in the supplementary data associated with the online version of this journal. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2005.09.015.

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