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Short communication

Novel ring-expanded nucleoside analogs exhibit potent and selective inhibition of hepatitis B virus replication in cultured human hepatoblastoma cells

Ramesh K. Sood [a], Vishweshwar S. Bhadti [a,d], Ali I. Fattom [a], Robert B. Naso [a], Brent E. Korba [b], Earl R. Kern [c], Huan-Ming Chen [d], Ramachandra S. Hosmane [d,*]

[a] *W.W. Karakawa Microbial Pathogenesis Laboratory, Nabi, Rockville, MD 20852, USA*
[b] *Division of Molecular Virology and Immunology, Georgetown University Medical Center, Rockville, MD 20850, USA*
[c] *Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL 35294, USA*
[d] *Laboratory for Drug Design and Synthesis, Department of Chemistry and Biochemistry, University of Maryland, Baltimore County (UMBC), 1000 Hilltop Circle, Baltimore, MD 21250, USA*

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Abstract

Novel ring-expanded nucleoside (REN) analogs (**1**–**3**) containing 5:7 fused ring systems as the heterocyclic base were found to be potent and selective inhibitors of hepatitis B virus (HBV) replication in cultured human hepatoblastoma 2.2.15 cells. The most active compound, 6-amino-4,5-dihydro-8*H*-1-(β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (**1**), inhibited the synthesis of intracellular HBV replication intermediates and extracellular virion release in 2.2.15 cells with 50% effective concentration (EC_{50}) of 0.604 and 0.131 μM, respectively. All three compounds had no effect on the synthesis of viral ribonucleic acids (RNA) in 2.2.15 cells. These compounds also exhibited low cellular toxicity in stationary and rapidly growing cell systems.

Keywords: Ring-expanded nucleoside analogs; In vitro HBV inhibition; Human hepatoblastoma cell line; Low toxicity in stationary and fast-growing cell systems

Hepatitis B virus (HBV) infections continue to be a major worldwide health problem (CDC, 1995). HBV infection is known to cause acute and chronic liver hepatitis, which could lead to liver cirrhosis. Worldwide there are an estimated 350 million chronic carriers of HBV and 1–2% of them die each year from virus related complica-

* Corresponding author. Tel.: +1-410-455-2520; fax: +1-410-455-1148.
E-mail address: hosmane@umbc.edu (R.S. Hosmane).

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tions. Chronic carriage of HBV has also been strongly associated with hepatocellular carcinoma (Beasley and Hwang, 1991).

A number nucleoside analogs have been shown to inhibit the replication of HBV in cell cultures and in animal models (Chu et al., 1995; Doong et al., 1991; Genovesi et al., 1998; Innaimo et al., 1997; Korba and Boyd, 1996; Lin et al., 1994a,b; Nicoll et al., 1998; Yokota et al., 1991; Zhu et al., 1997; Colacino and Staschke, 1998). More recently, 2′,3′-dideoxy-L-3′-thiacytidine (3TC) has become the first and the only nucleoside analog that has been approved for the treatment of chronic HBV infection in humans. Several other pyrimidine and purine containing nucleoside analogs with either a modified ribose or an acyclic alkyl chain as the sugar moiety have been shown to exhibit anti-HBV activity (Lin et al., 1994a,b; Colacino and Staschke, 1998). Many of these nucleoside analogs are currently being evaluated against HBV infections in humans (Bowden, 1997). In the majority of these nucleosides with anti-HBV activity, the sugar part of the molecule is modified to make them inhibitors of the viral polymerases. Some of the modifications which impart antiviral activity to these compounds include changing the sugar configuration to that of an L-enantiomer and/or removal and replacement of the 2′,3′-hydroxyls, e.g. 3TC; substitution of cyclic ribose with an acyclic side chain as in acyclic phosphonate analogs; or removal of ring oxygen as in carbocyclic analogs. We have prepared a series of modified purine analogs where the six membered ring of the natural purine heterocycle has been expanded to a seven membered ring. The newly synthesized ring expanded heterocycles in compound **1** and **2** still maintain their planarity and aromaticity, the two characteristics which are common with natural purines adenosine and guanosine. Furthermore, because of their ability to base-pair with natural pyrimidine bases, as suggested by our preliminary molecular modeling and ^{1}H-NMR studies, ring-expanded nucleosides (RENs) may potentially be incorporated into nucleic acids, provided they are appropriately phosphorylated in vivo by kinases. In this study, we report the anti-HBV activity of three RENs. The three nucleosides **1**–**3** reported here are selective inhibitors of intracellular HBV replication and extracellular HBV virion synthesis in chronically HBV-producing hepatoblastoma cells 2.2.15. These compounds also exhibit very low cellular toxicity in stationary and rapidly growing cell systems.

The three REN analogs, 6-amino-4,5-dihydro-8*H*-(1-β-D-ribofuranosyl)imidazo[4,5-*e*][1,3]diazepine-4,8-dione (**1**), 4,6-diamino-8*H*-8-imino-1-(β-D-ribofuranosyl)imidazo[4,5-*e*] [1,3]diazepine (**2**) and 5,6,7,8-tetrahydro-4*H*-1-(β-D-ribofuranosyl)-imidazo[4,5-*e*] [1,2,4]triazepine-5,8-dione (**3**) were synthesized according to the procedures published previously (Chen et al., 1999; Bhadti et al., 1992; Wang et al., 1994) (Fig. 1).

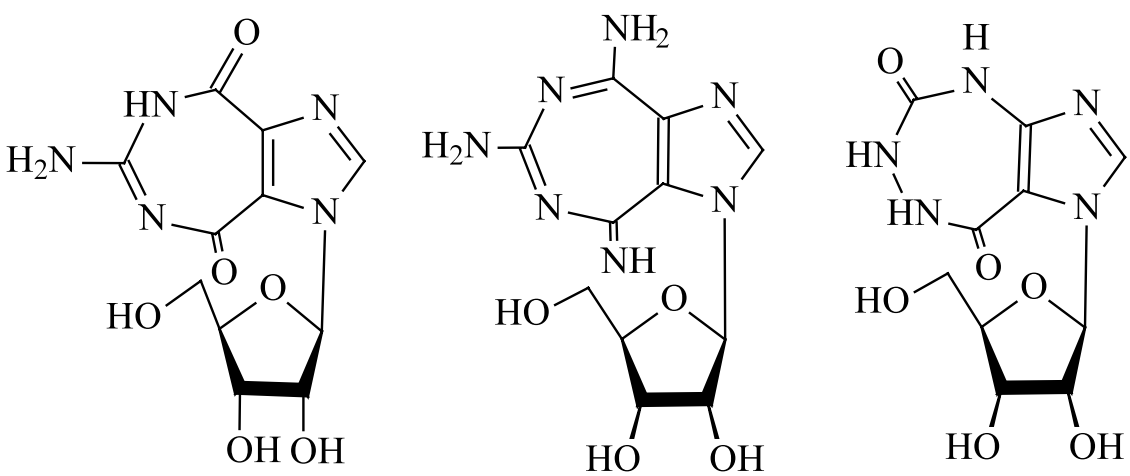
Anti-HBV activity and the toxicity against confluent 2.2.15 cells were determined by the previously described procedure (Korba and Gerin, 1992). Briefly, confluent cultures of HBV transfected 2.2.15 cells were maintained on 24-well flat-bottomed tissue culture plates in RPMI 1640 medium with 2% fetal bovine serum (FBS). The cells were treated with test compounds daily for 9 consecutive days. Samples of the culture medium were taken for analysis on day 0, 3, 6, and 9 to quantify the amount of HBV virion DNA. On day 10, the cells were lysed to quantify the amount of intracellular HBV replicative intermediates (RI). The quantification of virion DNA and the HBV RI were done by blot hybridization methods (southern or dot blot). Cellular toxicity against confluent 2.2.15 cells was measured after treatment with the compounds for 9 days as described above for antiviral activity experiments and comparing the uptake of neutral red dye by treated cells with untreated control cells.

Toxicity of compounds **1**–**3** was also measured in rapidly growing human foreskin fibroblast (HFF) cells and Daudi cells by cell proliferation assay. The appropriate cells were seeded in six well plates in MEM containing 10% FBS. Twenty-four hours later, the cells were treated with several dilutions of the test compounds covering a range of 100–0.03 μg/ml. The plates were incubated in a CO_2 incubator at 37 °C for 72 h. The cells were washed after removing the media and subsequently released from the plate wells by

adding 1 ml of 0.25% trypsin. The contents in the wells were mixed, diluted with Isotone III and counted using a coulter counter. The CC_{50} values (concentration of compound giving 50% inhibition of cell proliferation) were calculated by linear regression analysis. Toxicity of compound **1** was also evaluated in bone marrow precursor cells by previously published procedure (Sommadossi et al., 1992).

All three compounds exhibited moderate to potent in vitro activity against HBV as shown in Table 1. Compounds **1**–**3** were able to inhibit the synthesis of extracellular HBV virions and intracellular HBV DNA RI in 2.2.15 cells following 9 days treatment with these compounds. Compound **1** was found to be the most active compound against HBV. The EC_{50} value of compound **1** for virion DNA inhibition was approximately 2–3-fold higher than 3TC, which has been recently approved in the United States and several other countries as a therapeutic agent against chronic HBV infections. This assay was repeated three times and the average EC_{50} and EC_{90} values for HBV virion inhibition obtained were 0.131 and 0.989 μM, respectively. Compounds **2** and **3**, although less active than compound **1**, were also able to inhibit the extracellular HBV virion synthesis in 2.2.15 cells. Comparison of the antiviral activity of **1** and **2** shows that replacement of the amino groups on the seven membered heterocycle with oxygen increased the in vitro anti-HBV activity by 3-fold. More importantly, this change in the structure resulted in a decrease in the in vitro cellular toxicity of compound **1** by 5-folds compared with toxicity of compound **2** in confluent 2.2.15 cells. It is interesting to note that all three compounds showing antiviral activity are riboside analogs. 2′-Deoxy analogs of compound **1** and **2** were found to have no activity against HBV (data not shown). We are currently studying the structural requirements of these compounds for optimization of their antiviral activities.

Next, we evaluated these compounds for their ability to inhibit viral ribonucleic acids (RNA) synthesis in 2.2.15 cells. Since HBV uses host cellular RNA polymerase II for the transcription of viral RNA from the covalently closed circular HBV DNA during its replication, any effect on the synthesis of viral RNA by these compounds would mean interference with the cellular RNA polymerase which could lead to unacceptable cellular toxicity. As shown in Table 2, like 3TC, all three compounds showed no inhibition of the synthesis of viral 3.6 and 2.1 kb RNA by HBV in 2.2.15 cells. In spite of no suppression of viral RNA synthesis in the presence of these com-



1 **2** **3**

Fig. 1. Chemical structures of ring-expanded nucleosides **1**–**3**.

Table 1
Antiviral activities of test compounds against HBV replication in 2.2.15 cells

Compounds	CC_{50} (μM)[a]	EC_{90} (μM)[b]		EC_{50} (μM)[b]		Selectivity Index[e]	
		Virion[c]	HBV RI[d]	Virion[c]	HBV RI[d]	Virion	HBV RI
1	2427	0.989 ± 0.107	7.3 ± 0.7	0.131 ± 0.019	0.604 ± 0.07	18 526	4018
2	501	4.7 ± 0.5	–	0.397 ± 0.028	–	1262	–
3	1844	14 ± 1	–	4.2 ± 0.3	–	439	
3TC	2039	0.198 ± 0.02	0.922 ± 0.085	0.065 ± 0.008	0.172 ± 0.019	31 863	11 854

Appropriate concentrations of the test compounds were added daily for 9 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 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.

[a] CC_{50} is the drug concentration at which a 2-fold reduction of neutral red dye uptake from the average value in the untreated cultures was observed.

[b] EC_{90} and EC_{50} are concentrations which give 90% and 50% inhibition of viral replication in the cell cultures, respectively. Values presented ($\pm$ S.D.) were calculated by linear regression analysis using data combined from all treated cultures; S.D. were calculated by using the S.E. of regression generated from the linear regression analysis.

[c] Extracellular HBV virion DNA.

[d] Intracellular HBV DNA RI.

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

pounds, it was interesting to see that treatment of 2.2.15 cells with these compounds, unlike 3TC, did result in the reduction of viral protein synthesis especially that of the core antigen. The significance of this observation is not clear at the present time.

The antiviral activity exhibited by compounds **1**–**3** was found to be specific against HBV. These compounds were also tested against HIV, herpes simplex virus (HSV)-1, HSV-2, cytomegalovirus (CMV), Varicella Zoster virus (VZV) and Epstein–Barr virus (EBV). These compounds showed no antiviral activity against these viruses (data not shown).

In vitro cellular toxicity of compounds **1**–**3** was evaluated in both stationary and rapidly growing cell systems. Toxicity of compound **1** was also studied in bone marrow precursor cells (by erythroid burst forming units and granulocyte macrophage CFU). The toxicity results are given in Table 3. In bone marrow precursor cells, compound **1** had CC_{50} values which are comparable with those exhibited by 3TC. In rapidly growing human HFF cells and Daudi cells, all three compounds were found to be non-toxic up to 100 and 50 μM concentrations, respectively.

In summary, the ribosyl analogs of three novel ring-expanded purine ring systems were found to selectively inhibit HBV replication in 2.2.15 cells. All three compounds were also found to have low cellular toxicity in various stationary and rapidly growing cell systems. These nucleoside analogs are structurally unique compared with other nucleosides with anti-HBV activity. They all have an expanded ring system as heterocycle and the antiviral activity seems to be associated with only ribosyl analogs at present. Compound **1**, which was most potent in inhibiting HBV replication in 2.2.15 cells and has very low cellular toxicity, is a promising candidate for further development. The future of this compound as a clinical candidate for the treatment of HBV infection will depend on its pharmacological properties, which are currently under investigation in our laboratory.

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Table 2
Effect of RENs on the relative levels of HBV RNA and proteins in 2.2.15 cells

Compound (concentration)[a]	Virion DNA[d]	HBV RI[d]	RNA (3.6 kb)[b,d]	RNA (2.1 kb)[b,d]	HBsAg[c,d]	HBcAg[c,d]	HbeAg[c,d]
Untreated	100 ± 14	100 ± 8	100 ± 10	100 ± 12	100 ± 15	100 ± 10	100 ± 9
Compound **1** (10 μM)	4 ± 1 (3 ± 1)	15 ± 2 (9 ± 1)	89 ± 9 (94 ± 11)	96 ± 7 (102 ± 10)	67 ± 6 (92 ± 14)	75 ± 4 (94 ± 6)	50 ± 5 (90 ± 8)
Compound **2** (10 μM)	1 ± 1 (0)	6 ± 1 (5 ± 1)	103 ± 9 (97 ± 7)	96 ± 8 (119 ± 11)	108 ± 18 (88 ± 6)	109 ± 6 (100 ± 8)	8 ± 3 (95 ± 8)
Compound **3** (30 μM)	1 ± 1 (0)	8 ± 1 (5 ± 1)	99 ± 6 (97 ± 7)	90 ± 7 (119 ± 11)	118 ± 4 (88 ± 6)	97 ± 56 (100 ± 8)	10 ± 4 (95 ± 8)

Cultures were treated for 9 days as explained in Table 1. For each treatment, a total of four separate cultures were used for the analysis of each marker. The values reported are the levels of the indicated HBV markers at the end of the treatment period (day 9) expressed as a percentage (± S.D.) of the average levels in the control cultures.

[a] Concentrations of antiviral agents used in each case approximate EC_{90} to EC_{95} values of the individual agents against HBV DNA RI.

[b] HBV nucleic acid levels were measured by standard blot hybridization.

[c] HBV protein levels were measured by standard semi-quantitative EIA methods.

[d] The values given in the parentheses are for 3TC which was used as a control.

Table 3
In vitro toxicity of REN analogs in various cell systems

Cell system	Compound **1**		Compound **2**		Compound **3**		Reference drug	
	CC_{50}	IC_{50}	CC_{50}	IC_{50}	CC_{50}	IC_{50}	CC_{50}	IC_{50}
Stationary 2.2.15 Cells	2427 μM	NA	501 μM	NA	1844 μM	NA	2039 μM (3TC)	NA
Stationary HFF Cells	>100 μM	NA	>100 μM	NA	>100 μM	NA	100 μM (ACV)	NA
Dividing HFF Cells	NA	>100 μM	NA	>100 μM	NA	>100 μM	NA (GCV)	40 μM
Dividing Daudi Cells	NA	>50 μM	NA	>50 μM	NA	>50 μM	NA (ACV)	>50 μM
BFU-E	NA	58 μM	NA	NT	NA	NT	NA (3TC)	69 μM
CFU-GM	NA	52 μM	NA	NT	NA	NT	NA (3TC)	60 μM

CC_{50}, cell cytotoxicity for stationary cells; IC_{50}, inhibition of cell proliferation in dividing cells; NT, not tested; NA, not applicable; ACV, acyclovir; GCV, ganciclovir; 3TC, lamivudine.

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