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Antiviral activity of 2,3′-anhydro and related pyrimidine nucleosides against hepatitis B virus

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ABSTRACT

Various 2,3′-anhydro analogs of 5-substituted 1-(2-deoxy-β-D-lyxofuranosyl)uracils (**10**–**15**) and a related 1-(3-*O*-mesyl-2-deoxy-β-D-lyxofuranosyl) pyrimidine nucleoside analog (**18**) have been synthesized for evaluation as a new class of potential anti-HBV agents. The compounds **10**, **12**, and **15** demonstrated most potent anti-HBV activities against duck HBV (DHBV) and human HBV with EC_{50} values in the range of 2.5–10 and 5–10 μg/mL, respectively, at non-toxic concentrations (CC_{50} = >200 μg/mL). The nucleoside **18** also demonstrated significant anti-HBV activity against DHBV with an EC_{50} value of 2.5 μg/mL, however, it was less active against HBV in 2.2.15 cells (EC_{50} = >10 μg/mL).

Hepatitis B virus (HBV) is a major cause of acute and chronic hepatitis in humans. According to the World Health Organization (WHO), HBV infection is one of the top 10 leading causes of death due to infectious diseases.[1] There are ~400 million chronic carriers of HBV worldwide and 20–30% of them will die due to exacerbation of chronic liver diseases such as cirrhosis, hepatocellular carcinoma (HCC), and liver failure.[2] HCC is fifth among most common cancer in humans. The probability of developing chronic HBV infection is reciprocal to the age of acquiring the infection. HBV has been known to be transmitted perinatally, sexually, and parenterally.[2] At the present time, only six agents are available for the treatment of chronic HBV infection; immunomodulatory treatment Interferon-α, nucleoside/nucleotide antivirals lamivudine (LMV), adefovir dipivoxil (ADV), entecavir (ETV), telbivudine (LdT), and tenofovir (TDF).[3] It has been observed that suppression of the replication of HBV in the liver by antiviral drugs leads to improved liver pathology and decreased progression to liver cirrhosis and hepatocellular carcinoma, resulting in reduced mortality and morbidity.[4] However, the currently available therapy is inadequate due to development of adverse effects. Continued treatment with monotherapy leads to development of resistance.[3,5] In addition, the most common limitation with the current therapy is viral rebound to baseline or even higher viral levels following cessation of treatment.[6,7] This problem of virus re-appearance can be in part explained by the mechanism by which HBV maintains the infection at the level of hepatocytes. It has been shown that chronic infection of hepatocytes is maintained by the presence of 30–40 copies of episomal covalently closed circular (ccc) viral DNA in the hepatocyte nucleus.[8] Viral cccDNA has a long half-life, is the template for virus transcription and is replenished by viral DNA synthesis. However, it can be expected that continuous and complete suppression of HBV DNA replication may deplete cccDNA.[9,10] Short-term therapy with HBV DNA synthesis inhibitors cannot deplete the pool of cccDNA, and this is the reason for rapid rebound of viral replication after cessation of therapy.[7,9,10]

The two major problems of current therapy; development of resistance and viral rebound may be addressed by continuous long-term therapy with potent non-toxic, non-cross-resistant anti-HBV agents or combination of anti-HBV agents. Substantive and continued suppression of viral DNA polymerase/RT may lead to continuous inhibition of viral DNA synthesis resulting eventually in the depletion of the cccDNA pool.[10] Thus, there is an urgent need to investigate additional new classes of antiviral agents for HBV infections. Although anti-HBV nucleos(t)ides target HBV polymerase, their specific modes of action can differ and may involve anti-priming, anti-reverse transcriptase, anti-DNA dependent DNA polymerase and/or combination of these mechanisms.[3] Also, cross-resistance between nucleos(t)ides does not necessarily occur.[3,4] These two attributes reinforce the notion that the search for novel anti-HBV agents must be continued and intensified.

We recently identified lyxofuranosyl pyrimidine nucleosides as a new class of potential anti-HBV agents where 1-(2′-deoxy-β-D-lyxofuranosyl)thymine (**1**) and 1-(2′-deoxy-β-D-lyxofuranosyl)-5-trifluoromethyluracil (**2**) exhibited appreciable in vitro inhibition of HBV in 2.2.15 cells (EC_{50} = 10 μg/mL) at non-toxic concentrations (CC_{50} = >200 μg/mL). In contrast 1-(2′-deoxy-β-D-lyxofuranosyl)-4-thiothymine (**3**) was inactive.[11] As part of our ongoing

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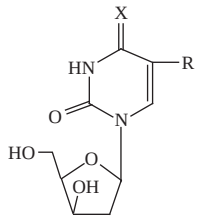
investigation of novel anti-HBV agents, it was of interest to discover more potent anti-HBV agents with low or no cytotoxicity.



1, R = CH_3 X=O

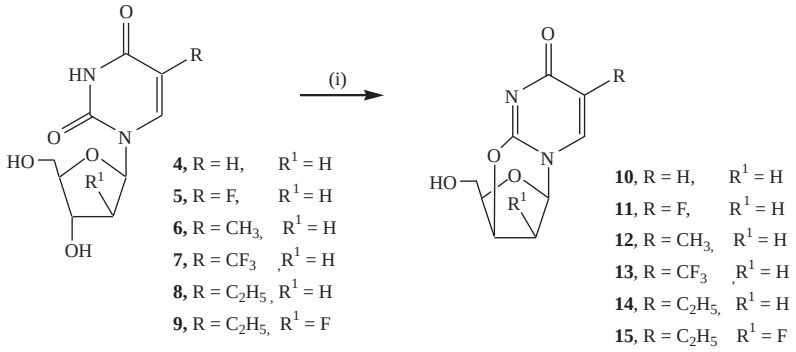
2, R = CF_3 X=O

3, R = CH_3 X=S

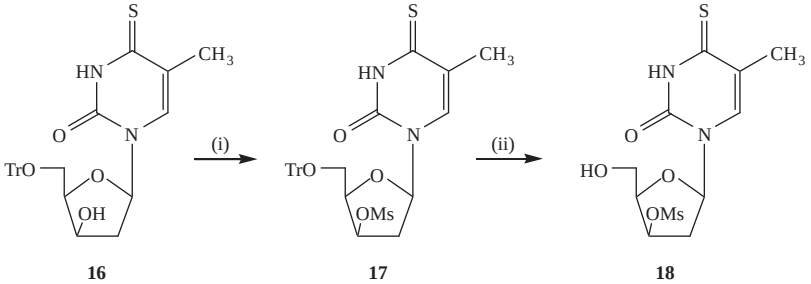
Pyrimidine nucleosides with an additional linkage between uracil base and carbohydrate portion, termed as anhydro or cyclo nucleosides have been studied in earlier investigations in an attempt to find more potent antiviral and antitumor agents,[12,13] for example, 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (cycloC) is a highly effective antitumor agent in animal models[12] and humans with toxicity less than that of its parent compound (araC).[13] CycloC acts as a depot form and slowly hydrolyzes to araC under physiological conditions.[12] 2,5′-Anhydro analogs of pyrimidine nucleosides have also been investigated in an effort to increase the antiviral activity and decrease the cytotoxicity.[14] However, to our knowledge, 2,3′-anhydro pyrimidine nucleosides have not been explored for biological evaluation as potential anti-HBV agents. In this Letter, we have investigated anti-HBV activities of various 5-substituted 2,3′-anhydro pyrimidine nucleosides (**10–15**) and a related analog (**18**) as potential antiviral agents. In analogy with cycloC, it is postulated that they may possess improved cellular penetration, physiological distribution and absorption, and undergo enzymatic hydrolysis in cell culture or in vivo to parent nucleosides.

The test compounds 2,3′-anhydro-1-(2-deoxy-β-D-lyxofuranosyl)uracil (**10**),[15] 2,3′-anhydro-1-(2-deoxy-β-D-lyxofuranosyl)-5-fluorouracil (**11**),[15] 2,3′-anhydro-1-(2-deoxy-β-D-lyxofuranosyl)-thymine (**12**),[15] 2,3′-anhydro-1-(2-deoxy-β-D-lyxofuranosyl)-5-trifluoromethyluracil (**13**),[16] 2,3′-anhydro-1-(2-deoxy-β-D-lyxofuranosyl)-5-ethyluracil (**14**),[15] and 2,3′-anhydro-1-(2-deoxy-2-fluoro-β-D-lyxofuranosyl)-5-ethyluracil (**15**) were synthesized by the reaction of nucleosides **4–9** with triphenylphosphine and diisopropyl azodicarboxylate in dry CH_3CN using the procedure reported by Balagopala et al.[17] (Scheme 1). The products were purified on silica gel column chromatography and structural assignments of the synthesized derivatives were made based on ^1H NMR and elemental analysis studies. In addition to anhydro compounds **10–15**, we also synthesized 1-(3-*O*-mesyl-2-deoxy-β-D-lyxofuranosyl)-4-thiothymine (**18**) as illustrated in Scheme 2 to investigate its potential activity against HBV. 1-(5-*O*-Trityl-2-deoxy-β-D-lyxofuranosyl)-4-thiothymine (**16**)[18] upon treatment with mesyl chloride in dry pyridine gave 1-(3-*O*-mesyl-5-*O*-trityl-2-deoxy-β-D-lyxofuranosyl)-4-thiothymine (**17**), which was detritylated to yield **18**[19] in 52% yield (Scheme 2).

The anti-HBV activity of nucleoside analogs **10–15** and **18**, along with the reference antiviral compound (−)3-TC was assessed in confluent cultures of primary duck hepatocytes obtained from chronically infected Pekin ducks according to the procedure previously reported by us.[20,21] Duck hepatitis B virus (DHBV), a member of hepadnaviridae, shares properties of hepatotropism, virion structure, genome organization and replication with human HBV.[22] DHBV-based in vitro and in vivo systems have been used extensively to screen drugs for potential anti-HBV activity.[23] It has been shown that compounds like 3-TC and penciclovir, both potent inhibitors of DHBV, are also potent inhibitors of HBV in chimpanzees as well as humans.[23,24] The anti-HBV activity of the compounds **10–15** and **18** was also assessed in confluent cultures of the human hepatoma cell line 2.2.15 that chronically produces infectious HBV. The anti-HBV activity data of compounds **4–9** in 2.2.15 cells is also included for comparisons. 2.2.15 is a stable human HBV-producing human hepatoblastoma cell line, which carries HBV DNA stably integrated into the genome of HepG2 cells.[25,26] Table 1 shows the concentrations required to inhibit 50% of HBV DNA (EC_{50}) and 50% cytotoxic concentration (CC_{50}) on Huh-7 cells.

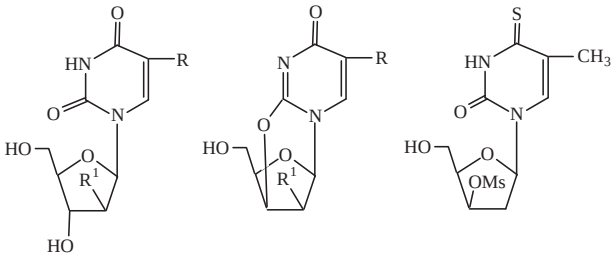


Scheme 1. Reagents and conditions: (i) triphenylphosphine, diisopropyl azodicarboxylate, dry CH_3CN, −15 °C to 0 °C, 2–5 h.



Scheme 2. Reagents and conditions: (i) mesyl chloride, dry pyridine, 0–5 °C, 40 h; (ii) 80% AcOH, 90 °C, 0.5 h.

Table 1
In vitro antiviral activities of pyrimidine nucleosides against duck HBV and human HBV replication



4-9 **10-15** **18**

Compd	R	R^1	DHBV primary duck hepatocytes % inhibition @ 10 μg/mL[a]	EC_{50}[b] (μg/mL)	2.2.15 HBV % inhibition @ 10 μg/mL[a]	EC_{50}[b] (μg/mL)	Cytotoxicity CC_{50}[e] (μg/mL)
4	H	H	ND[f]	—	0	—	>200
5	F	H	ND	—	ND	—	ND
6	CH_3	H	ND	—	0	—	>200
7	CF_3	H	ND	—	0	—	>100
8	C_2H_5	H	ND	—	40	>10	>200
9	C_2H_5	F	ND	—	90	<10	>200
10	H	H	67	2.5–5	65	5	>200
11	F	H	30	—	0	—	>200
12	CH_3	H	56	10	54	10	>200
13	CF_3	H	35	>10[c]	25	—	>200
14	C_2H_5	H	32	—	51	10	>200
15	C_2H_5	F	65	2.5–5	53	10	>200
18	—	—	70	2.5	45	>10	>200
3-TC[d]	—	—	96 (0.5–1.0)	0.05	88 (0.5–1)	0.2	>200

[a] The data is expressed as percent inhibition of viral DNA in the presence of 10 μg/mL of the test compounds as compared to untreated infected controls.
[b] The drug concentration (μg/mL) required to reduce the viral DNA in infected cells to 50% of untreated infected controls.
[c] (>) sign indicates that 50% inhibition was not reached at the stated (highest) concentration tested.
[d] (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (Lamivudine, 3-TC).
[e] Concentration required to reduce Huh-7 cell viability by 50%.
[f] Not determined.

Among the synthesized compounds, **10**, **12**, **15**, and **18** elicited significant activity against DHBV with EC_{50} values of 2.5–5, 10, 2.5–5, and 2.5 μg/mL, respectively. Compounds **11**, **13**, and **14** were found to provide only 30–35% inhibition at 10 μg/mL concentration. In these studies, we noted that incorporation of a fluorine atom at the 2′-arabino position of **14** led to an increased DHBV inhibition (as compared to compound **15**). When the test compounds **10**–**15** and **18** were evaluated against HBV in 2.2.15 cells, only nucleosides **10**, **12**, and **13** retained their activity, **15** and **18** had reduced inhibition of viral replication, and **11** lost the inhibitory action. In this system, on the other hand, compound **14** showed increased activity (EC_{50} = 10 μg/mL) as compared to its anti-DHBV activity (32% inhibition @ 10 μg/mL). The marked differences obtained between DHBV and HBV activities could be attributed to inherent differences in human versus duck HBV, metabolic differences between the two cells and/or genome organization of hepadnavirus (i.e., integrated in 2.2.15 cells and non-integrated in duck hepatocytes).

It was interesting to note that in 2.2.15 cells, 2,3′-anhydro analog (**12**) was almost as active as its lyxo (non-anhydro) derivative (**1**, EC_{50} = 10 μg/mL),[11] and the 2,3′-anydro derivative (**13**) was much less active (25% inhibition @ 10 μg/mL) than the lyxo analog (**2**, EC_{50} = 10 μg/mL).[11] In contrast, 2,3′-anhydro-1-β-D-lyxofuranosyluracil (**10**) (EC_{50} = 5 μg/mL) and 2,3′-anhydro-1-β-D-lyxofuranosyl-5-ethyluracil (**14**) (EC_{50} = 10 μg/mL) gained significant activity compared to their 3′-OH (lyxo) derivatives that proved to be completely inactive in our previous studies.[11] In line with the observations made with 2,3′-anhydro analogs **10** and **14**, we were interested to examine if blocking the 3′-OH lyxo group in compound **3** by other means could enhance anti-HBV activity. It was noteworthy that incorporation of a mesyl group at the 3′-position [as in **18**, EC_{50} = 2.5 μg/mL (DHBV) and 45% inhibition @ 10 μg/mL (2.2.15 cells)] also influenced the antiviral activity dramatically since its non-mesylated compound (**3**) had no anti-HBV activity in our earlier studies.[11] Reasons for the increase and decrease in activities of the compounds compared to the parent analogs are not clear at this point. However, our results indicate that the modifications studied in this Letter can lead to the modulation of the anti-HBV activity in in vitro, possibly due to changes in properties such as cell delivery, slow release, and/or resistance against enzymatic hydrolysis.

Hydrolytic stability of compounds **10–15** was determined in saline and PBS buffer at physiological pH at 25 °C and 37 °C up to 48 h, and in fetal bovine serum at 37 °C for 24 h. No conversion to parent nucleosides (**4–9**), lyxo (3′-OH up) derivatives or degradation products was obtained under these conditions. The potent anti-HBV activity of compounds **10**, **12**, and **14** as compared to their parent nucleosides **4**, **6**, and **8** and decreased inhibition of HBV by **15** as compared to its parent analog **9** suggests that 2,3′-anhydro nucleosides (**10–15**) possess anti-HBV activity on their own.

The fact that nucleosides **10**, **12**, **14**, **15**, and **18** exhibiting anti-DHBV or anti-HBV activity in a human hepatoma cell line carrying the HBV (2.2.15 cells) and/or against both viruses show no host cell cytotoxicity (CC_{50} = >200 μg/mL), suggests that they can selectively inhibit HBV DNA polymerases after phosphorylation by cellular kinases. Similar mechanisms of action have been suggested for other antiviral nucleosides.[24]

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16. Experimental synthesis of 2,3′-anhydro-1-(2-deoxy-β-D-lyxofuranosyl)-5-trifluoromethyl uracil (**13**). A dried mixture of 5-trifluoromethyl-2′-deoxyuridine (**7**; 1.0 g, 3.38 mmol) and triphenylphosphine (1.78 g, 6.79 mmol) was suspended in acetonitrile (50 mL) and the reaction mixture was cooled to −15 °C in ice-methanol bath. Diisopropylazodicarboxylate (1.32 mL, 6.73 mmol) was slowly added with vigorous stirring in 10 min, maintaining the reaction temperature below −5 °C. The reaction mixture was then stirred at 0 °C for 3 h. The solvent was removed in vacuo and the residue obtained was purified by elution from a silica gel column using MeOH/$CHCl_3$ (15:85, v/v) as eluent to give **13** (0.63 g, 67%) as a white solid; mp 190–192 °C. ^{1}H NMR (DMSO-d_6) δ 2.54 and 2.68 (2 m, 2H, H-2′), 3.54 (t, J = 5.80 Hz, 2H, H-5′), 4.24 (m, 1H, H-4′), 5.07 (t, J = 5.50 Hz, 1H, 5′-OH), 5.36 (br s, 1H, H-3′), 6.04 (d, J = 3.66 Hz, 1H, H-1′), 8.46 (s, 1H, H-6). Anal. ($C_{10}H_9F_3N_2O_4$) C, H, N.
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19. Experimental synthesis of 1-(3-*O*-mesyl-2-deoxy-β-D-lyxofuranosyl)-4-thiothymine (**18**). To an ice cooled solution of 1-(5-*O*-trityl-2-deoxy-β-D-lyxofuranosyl)-4-thiothymine (**16**, 0.5 g, 0.99 mmol) in dry pyridine (20 mL) was added mesyl chloride (0.13 mL, 1.68 mmol) drop wise with stirring. Then, the reaction mixture was kept in the refrigerator for 40 h. After the addition of water (1 mL), the solvent was evaporated and the resulting residue was dissolved in $CHCl_3$ (50 mL), washed with water (2 × 25 mL) and dried over anhydrous Na_2SO_4. The solvent was removed in vacuo and the residue thus obtained was purified on a silica gel column using MeOH/$CHCl_3$ (2:98, v/v) as eluent to give **17** (0.22 g, 38%) as a sirup. ^{1}H NMR ($CDCl_3$) δ 1.98 (s, 3H, CH_3), 2.49–2.58 (m, 1H, H-2′), 2.74–2.88 (m, 1H, H-2″), 2.77 (s, 3H, CH_3), 3.34–3.42 (m, 1H, H-5′), 3.63–3.71 (m, 1H, H-5″), 4.27 (m, 1H, H-4′), 5.29 (m, 1H, H-3′), 6.23 (dd, J = 7.94 Hz, 2.75 Hz, 1H, H-1′), 7.24–7.52 (m, 16H, 5′-*O*-trityl and H-6), 10.05 (br s, 1H, NH). A solution of **17** (0.20 g, 0.34 mmol) in 80% aqueous AcOH (20 mL) was heated at 90 °C for 0.5 h. The solvent was removed in vacuo and the crude product thus obtained was purified on a silica gel column using EtOAc/hexane (70:30, v/v) as eluent to give **18** (0.06 g, 52%) as a solid; mp 136–138 °C. ^{1}H NMR (DMSO-d_6) δ 1.98 (s, 3H, CH_3), 2.35 (dd, J = 15.56 Hz, 2.13 Hz, 1H, H-2′), 2.81–2.91 (m, 1H, H-2″), 3.23 (s, 3H, CH_3), 3.73 (t, J = 5.49 Hz, 2H, H-5′), 4.10–4.15 (m, 1H, H-4′), 5.06 (t, J = 5.49 Hz, 1H, 5′-OH), 5.27 (t, J = 4.28 Hz, 1H, H-3′), 6.09 (dd, J = 7.93 Hz, 2.75 Hz, 1H, H-1′), 7.56 (s, 1H, H-6), 12.77 (s, 1H, NH). ^{13}C NMR (CD_3OD) δ 17.43 (CH_3), 38.31 (CH_3), 40.63 (C-2′), 60.52 (C-5′), 80.54 (C-3′), 84.66 (C-4′), 86.04 (C-1′), 120.19 (C-5), 133.14 (C-6), 149.82 (C-2), 192.51 (C-4). Anal. ($C_{11}H_{16}N_2O_6S_2$) C, H, N, S.
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