Effect of Various Pyrimidines Possessing the 1-[(2-Hydroxy-1-(hydroxymethyl)ethoxy)methyl] Moiety, Able To Mimic Natural 2′-Deoxyribose, on Wild-type and Mutant Hepatitis B Virus Replication

Rakesh Kumar,*,† Wassila Semaine,†,‡ Monika Johar,† D. Lorne J. Tyrrell,§ and Babita Agrawal*,‡

Department of Laboratory Medicine and Pathology, 1-41 Medical Sciences Building, Department of Surgery, and Department of Medical Microbiology and Immunology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, AB, Canada T6G 2H7

Received January 31, 2006

Hepatitis B virus (HBV) is the most common cause of chronic liver disease worldwide. Development of drug resistance against clinical anti-HBV drug lamivudine due to long-term use and rebound of viral DNA after cessation of treatment has been a major setback of the current therapy. We have synthesized a series of pyrimidine nucleosides possessing a variety of substituents at the C-5 position, and a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] flexible acyclic glycosyl moiety at the N-1 position, that have the ability to mimic the natural 2′-deoxyribosyl moiety. Some of these potential antiviral compounds included variations at both C-5 and C-6 positions of the uracil base. Other variations of the uracil derivatives were the 6-aza congeners. 4-Amino and 4-methoxy pyrimidine derivatives were also made. Compounds in which the base moiety was substituted by 5-chloro- (**25**), 5-(2-bromovinyl)- (**32**), or 5-bromo-6-methyl- (**37**) groups possess significant activity against duck-HBV, wild-type human HBV (2.2.15 cells), and lamivudine-resistant HBV containing single and double mutations. No cytotoxicity was seen in host HepG2 and Vero cells, up to the highest concentration tested. The anti-HBV activity exhibited by compounds **25**, **32**, and **37** was superior for human HBV and comparable for DHBV to that of the corresponding purine nucleoside, ganciclovir. Further, they were only 10−15-fold less inhibitory against human HBV in 2.2.15 cells than the reference drug, lamivudine. Other compounds in the series were moderately inhibitory against DHBV and wild-type human HBV. The size of the halogen and the electronegativity of the substituents at the 5- and 6-positions are important for antiviral activity toward HBV. These compounds were also evaluated for their antiviral activity for West Nile virus, respiratory syncytial virus, SARS-coronavirus, and hepatitis C virus. They were generally inactive in these antiviral assay systems (at concentrations up to 100 μg/mL). 1-[(2-Hydroxy-1-(hydroxymethyl) ethoxy)methyl]-5-fluorocytosine (**34**) showed some inhibitory activity against hepatitis C virus. Taken together, these data support our previous observations that the 5-substituted pyrimidine nucleosides containing acyclic glycosyl moieties have potential to serve as a new generation of potent, selective, and nontoxic anti-HBV agents for wild-type and lamivudine-resistant mutant HBV.

Introduction

Hepatitis B virus (HBV) infection is a significant problem affecting more than 5% of the world's population.[1−3] HBV infections are present in people in all parts of the world. HBV can cause acute and chronic liver diseases. Chronic HBV infection often results from exposure in early life, leading to viral persistence in the absence of a strong antibody or cellular immune response. There are over 350 million people worldwide who are chronic carriers of HBV, 6 times the number of people infected with human immunodeficiency virus (HIV) (50 million). Chronic HBV infection leads to liver damage, cirrhosis, and liver cancer with high mortality. It has been suggested that suppression of the replication of HBV in the liver leads to improved liver pathology and decreased progression to liver cirrhosis and hepatocellular carcinoma, both of which are secondary to HBV infection and major causes of mortality. Therapy of chronic HBV can aim to either inhibit viral replication or enhance immunological response against the virus, or both.

α-Interferon (α-IFN, Intron A) has been used for chronic HBV infections, but its use is limited. Less than one-half of all chronic HBV patients are eligible for this treatment. Unfortunately, of those who are treated, α-IFN therapy is effective only in 30−40% of the cases. It is expensive, requires i.v. administration, and has serious side effects such as influenza-like symptoms, depression, insomnia, nausea, abdominal pain, hair loss, leucopenia, thrombocytopenia, thyroid disorders, and rashes.[2,4,5] Thymosin-α_1 (Zadaxin), interleukin-12, and therapeutic vaccines have been used in clinical trials as immunotherapy regimens, but have not demonstrated sufficient efficacy.[6−8]

HBV is an incomplete double-stranded DNA virus. Its DNA replication is unique and includes a reverse transcriptase-catalyzed reverse transcription step.[9] The HBV DNA polymerase is an essential and multifunctional enzyme, which functions as a DNA polymerase, reverse transcriptase, RNAse H, coordinates the assembly of viral nucleocapsids, and catalyzes the generation of DNA primer. Therefore, different classes of compounds can interfere at various steps of HBV replication. Furthermore, HBV DNA polymerase is quite different from human DNA polymerases, suggesting that compounds that selectively inhibit HBV replication can be identified.[10]

The (−) enantiomer of 2′3′-dideoxy-3′-thiacytidine (**1a**), also known as lamivudine (3-TC), is a potent inhibitor of HBV replication.[11,12] It is phosphorylated by cellular kinases into the triphosphate form, which competes with cellular dCTP and is incorporated into viral DNA by the viral DNA polymerase. This results in premature chain termination during both the reverse

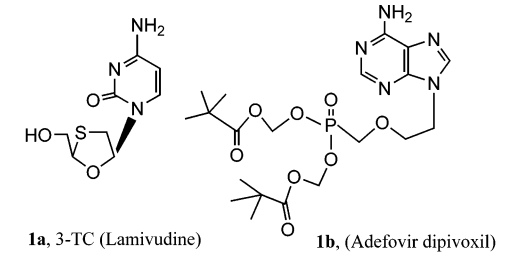
* Corresponding author. Phone: (780) 492-7545. Fax: (780) 492-7521. E-mail: rakesh.kumar@ualberta.ca.
† Department of Laboratory Medicine and Pathology.
‡ Department of Surgery.
§ Department of Medical Microbiology and Immunology.

10.1021/jm060102l CCC: $33.50
Published on Web 05/12/2006

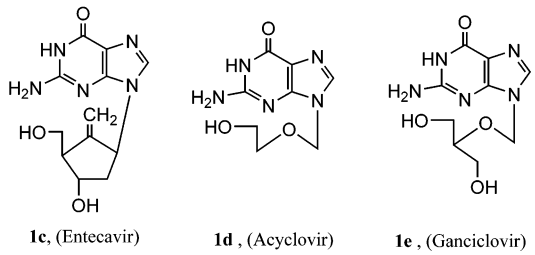
transcription and the DNA synthesis steps of HBV DNA synthesis.[13] Chronically infected individuals treated with lamivudine therapy show histological improvement and significant reduction of viral load.[14,15]

Recently another antiviral agent, hepsera, 9-[2-[bis[(pivaloyloxy)methoxy]phosphinyl]methoxy]-ethyl]adenine [adefovir dipivoxil] (**1b**), an oral prodrug of an acyclic nucleotide analogue of adenosine, has been approved by the FDA for HBV infection.[16−19] In this drug, after absorption, the bis-(pivaloyloxymethyl) moiety is removed from the parental drug, resulting in the active agent adefovir. Hepsera, being a monophosphate derivative, does not require initial phosphorylation to the monophosphate for activation.[16] Adefovir diphosphate is an inhibitor of HBV DNA polymerase (reverse transcriptase) activity and is also a chain terminator when incorporated into viral DNA. Hepsera was demonstrated to be well-tolerated, to reduce serum HBV DNA levels by 4 logs, and lead to seroconversion in 20−27% of the patients treated for 12 weeks at a daily dose of 30 mg or greater/day.[17,18] However, primary limitations of adefovir therapy are dose-related side effects such as nephrotoxicity, lactic acidosis, and severe hepatomegaly with steatosis, and cessation of therapy may result in serious hepatitis.[19]

More recently, a cyclopentyl deoxyguanosine analogue, baraclude, [1*S*-(1α,3α,4*β*)]-2-amino-1,9-dihydro-9-[4-hydroxy-3-3(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one] [entecavir] (**1c**), in which the oxygen in the furanose ring is replaced with an exocyclic double bond, has been approved by the FDA for HBV infection.[20] In in vitro studies, it is significantly more potent than lamivudine in HepG2 2.2.15 hepatoma cells with high therapeutic index and is also an effective inhibitor of replication of lamivudine-resistant HBV mutant.[21,22] Entecavir was found to be superior to lamivudine and also efficacious in patients developing resistance to lamivudine.[23,24] However, this drug also suffers from serious toxicity problems such as lactic acidosis and hepatotoxicity with hepatomegaly and steatosis.



1a, 3-TC (Lamivudine) **1b**, (Adefovir dipivoxil)



1c, (Entecavir) **1d** , (Acyclovir) **1e** , (Ganciclovir)

Treatment with lamivudine, hepsera, or baraclude in HBV-infected patients leads to a quantitative decrease in HBV replication. However, after cessation of treatment, virus DNA levels rebounded to baseline pretreatment (or even higher) levels.[25−27] This problem of virus reappearance can be in part explained by the mechanism by which HBV maintains the infection at the level of individual hepatocytes. It has been shown that chronic infection of hepatocytes is maintained by the presence of 30−40 copies of covalently closed circular (ccc) viral DNA in the hepatocyte nucleus. A drawback of lamivudine and hepsera therapy is that the template for hepadnavirus replication, cccDNA, is relatively unaffected.[28] Although the half-life of HBV cccDNA in humans has not been established, the half-lives of both duck HBV and woodchuck HBV cccDNA have been shown to be quite long, between 30 and 57 days.[29,30] Because of the stability of cccDNA, continuous suppression of viral replication will likely require long-term anti-HBV therapy.

Prolonged lamivudine monotherapy results in the development of lamivudine-resistant HBV variants in approximately 43% of treated patients within a year and 49−58% patients in 2−3 years.[31] The appearance of resistant HBV suggests that lamivudine therapy must be combined with other antiviral drugs to delay or prevent the emergence of resistance mutants. Thus, the future treatment of chronic HBV should encompass a cocktail of antiviral drugs as has been proven effective for anti-HIV therapy.

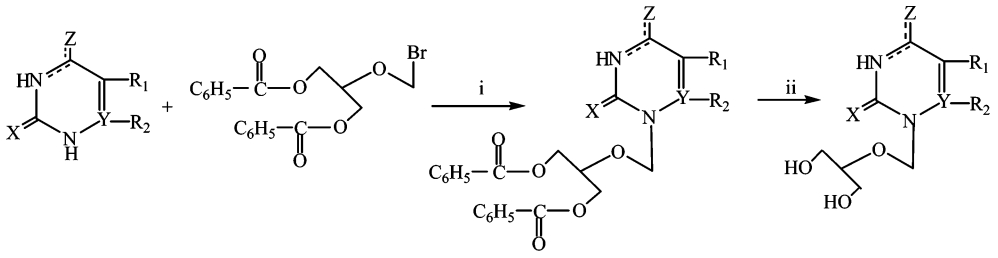
Mutations associated with lamivudine resistance occur most often in the conserved YMDD motif of the nucleotide-binding site of the viral DNA polymerase.[32−34] Single nucleotide changes at codon 204 of the reverse transcriptase domain of the polymerase results in the substitution of either valine or isoleucine for methionine (M204V or M204I). The valine substitution and occasionally the isoleucine substitution is accompanied by an additional upstream mutation at codon 180 (L180M).[32]

Molecular modeling studies suggested that acyclic nucleosides provide torsional flexibility and a shorter chain connecting the base and the sugar moiety, as compared to the oxathiolane ring in lamivudine. The molecular modeling studies also predicted that the dNTP binding pocket of HBV DNA pol containing M204V/I+L180M mutations is more constrained and crowded, allowing acyclic nucleoside analogues to be accommodated more effectively. This modeling prediction was confirmed with biochemical studies using HBV polymerase inhibition assays.[35] Therefore, nucleoside analogues that have sugar ring systems that are modified or acyclic may retain activity against lamivudine-resistant mutants.[35] This discrepancy in the mode of binding of nucleoside analogues with modified glycosyl moieties to the dNTP binding pocket of HBV DNA pol permits the possibility that lamivudine-resistant HBV could be treated with other nucleoside agents singly or in combination.

Among acyclic purine nucleosides, acyclovir 9-[(2-hydroxyethoxy)methyl]guanine (**1d**) has been shown to be a modest inhibitor of HBV DNA polymerase in triphosphate form. Acyclovir exhibited both in vitro and in vivo activity in animal models, but demonstrated disappointing anti-HBV efficacy in human clinical trials.[36] Ganciclovir, 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]guanine (**1e**), structurally similar to acyclovir but differing in the functional equivalent of a 3′-OH group, is also a potent inhibitor of HBV, both in cell culture against duck-HBV (DHBV) and in in vivo animal models.[37,38] However, long-term clinical use of ganciclovir is limited due to its severe dose-related toxicity.[39] In contrast, pyrimidine nucleosides with a 1-[(2-hydroxyethoxy)methyl] group have been explored as anti-herpes agent with no significant activity.[40−44]

Recent work in our laboratory has led to the identification of acyclic pyrimidine nucleosides as a new family of potent and selective inhibitors of DHBV replication.[45,46] More recently, various 5-substituted acyclic pyrimidines containing a 1-[(2-hydroxyethoxy)methyl] glycosyl moiety at the N-1 position showed potent in vitro activity in both chronically infected primary duck hepatocytes and a human HBV DNA transfected human hepatoblastoma cell line (HepG2 2.2.15).[47] Intriguingly,

Scheme 1[a]



2-20 **22-41**

2 = R_1 = H, R_2 = H, X = O, Y = C, Z = O
3 = R_1 = F, R_2 = H, X = O, Y = C, Z = O
4 = R_1 = Br, R_2 = H, X = O, Y = C, Z = O
5 = R_1 = Cl, R_2 = H, X = O, Y = C, Z = O
6 = R_1 = NO_2, R_2 = H, X = O, Y = C, Z = O
7 = R_1 = NH_2, R_2 = H, X = S, Y = C, Z = O
8 = R_1 = CH_3, R_2 = H, X = S, Y = C, Z = O
9 = R_1 = C_2H_5, R_2 = H, X = O, Y = C, Z = O
10 = R_1 = CH_2OH, R_2 = H, X = O, Y = C, Z = O
11 = R_1 = CH_2CH_2OH, R_2 = H, X = S, Y = C, Z = O
12 = R_1 = CH=CHBr, R_2 = H, X = O, Y = C, Z = O
13 = R_1 = H, R_2 = H, X = O, Y = C, Z = NH_2
14 = R_1 = F, R_2 = H, X = O, Y = C, Z = NH_2
15 = R_1 = Br, R_2 = H, X = OCH_3, Y = C, Z = OCH_3
16 = R_1 = H, R_2 = CH_3, X = O, Y = C, Z = O
17 = R_1 = Br, R_2 = CH_3, X = O, Y = C, Z = O
18 = R_1 = I, R_2 = CH_3, X = O, Y = C, Z = O
19 = R_1 = H, X = O, Y = N, Z = O
20 = R_1 = CH_3, X = O, Y = N, Z = O
21 = R_1 = CH_3, X = S, Y = N, Z = O

22 = R_1 = H, R_2 = H, X = O, Y = C, Z = O
23 = R_1 = F, R_2 = H, X = O, Y = C, Z = O
24 = R_1 = Br, R_2 = H, X = O, Y = C, Z = O
25 = R_1 = Cl, R_2 = H, X = O, Y = C, Z = O
26 = R_1 = NO_2, R_2 = H, X = O, Y = C, Z = O
27 = R_1 = NH_2, R_2 = H, X = S, Y = C, Z = O
28 = R_1 = CH_3, R_2 = H, X = S, Y = C, Z = O
29 = R_1 = C_2H_5, R_2 = H, X = O, Y = C, Z = O
30 = R_1 = CH_2OH, R_2 = H, X = O, Y = C, Z = O
31 = R_1 = CH_2CH_2OH, R_2 = H, X = S, Y = C, Z = O
32 = R_1 = CH=CHBr, R_2 = H, X = O, Y = C, Z = O
33 = R_1 = H, R_2 = H, X = O, Y = C, Z = NH_2
34 = R_1 = F, R_2 = H, X = O, Y = C, Z = NH_2
35 = R_1 = Br, R_2 = H, X = O, Y = C, Z = OCH_3
36 = R_1 = H, R_2 = CH_3, X = O, Y = C, Z = O
37 = R_1 = Br, R_2 = CH_3, X = O, Y = C, Z = O
38 = R_1 = I, R_2 = CH_3, X = O, Y = C, Z = O
39 = R_1 = H, X = O, Y = N, Z = O
40 = R_1 = CH_3, X = O, Y = N, Z = O
41 = R_1 = CH_3, X = S, Y = N, Z = O

[a] Reagents: (i) Hexamethyldisilazane, trimethylchlorosilane, potassium nonaflate, dry acetonitrile, reflux; (ii) 40% aqueous methylamine, 25 °C.

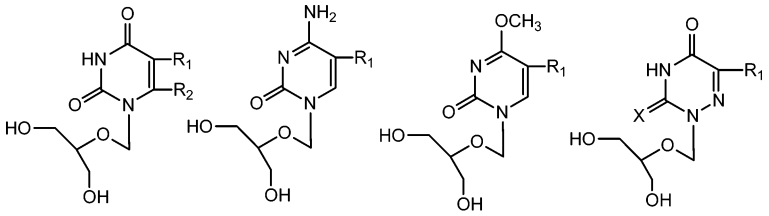
these compounds also exhibited in vitro activity against lamivudine-resistant HBV containing single and double mutations.[47] In our studies, we noted that, among acyclic pyrimidines, substituents at the C-5 position of the pyrimidine base are important determinants of anti-HBV activity against duck HBV, wild-type human HBV, and lamivudine-resistant HBV in vitro at noncytotoxic concentrations. It was therefore of interest to examine the anti-HBV effect of various 5-substituted acyclic pyrimidine nucleosides containing a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] moiety with a 3′-OH like group, to determine if they have increased affinity for viral DNA polymerase, thus providing improved anti-HBV activity and selectivity.

In an effort to further explore the structure activity relationships (SARs), we now report the synthesis and antiviral activities of several 5-substituted pyrimidines with a 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] acyclic chain. We herein also present modifications of the 4-, 5-, and 6- positions in this new series of promising antivirals and report their anti-HBV activity. The results presented here extend our previous observations and suggest the usefulness of the acyclic pyrimidine nucleosides as HBV inhibitors.

Chemistry

Acyclonucleosides **22**−**24**, **26**−**29**, **32**−**35**, and **39**−**41** were synthesized by the reaction of respective silylated uracils with bromomethyl ether as described earlier.[40−44] Similar synthetic routes for the preparation of unknown acyclic derivatives (**25**, **30**, **31**, and **36**−**38**) were adopted (Scheme 1). Thus, 5-substituted uracils (**5**, **10**, **11**) were refluxed with hexamethyldisilazane (HMDS) and a catalytic amount of chlorotrimethylsilane for about 3 h. The resulting clear solutions were evaporated in vacuo to remove excess HMDS, and the silylated intermediates obtained were coupled with 2-(bromomethoxy)-1,3-propenediyl dibenzoate[43] in dry acetonitrile at reflux temperature for 6−12 h to produce the precursor ester derivatives. These derivatives were subsequently deprotected with 40% aqueous methylamine at 25 °C to yield the target 1-[(2-hydroxy-1-(hydroxymethyl)-

Table 1. In Vitro Activity Against Wild-type (Duck HBV and Human HBV) and Drug-Resistant Hepatitis B Virus, and Toxicity of Various Acyclic Analogues of 2′-Deoxy Pyrimidine Nucleosides



22-32, 36-38 **33,34** **35** **39-41**

				% inhibition @10 μg/mL[a]		[EC$_{50}$ (μM)][b]		toxicity CC$_{50}$ (μM)	
no.	R_1	R_2	X	DHBV primary duck hepatocytes	2.2.15 wild-type HBV	B1 cell line M204I mutant HBV	D88 cell line L180M/M204V mutant HBV	HepG2 cells[c]	Vero cells[e]
22	H	H	O	0	0	ND[f]	ND	>462	>462
23	F	H	O	24 [>42.7]	26 [>42.7]	ND	ND	>427	>427
24	Br	H	O	48 [>33.8]	40 [>33.8]	ND	ND	>338	>338
25	Cl	H	O	69 [4−20]	60 [20−40]	56 [20−40]	40 [>40]	>400	>400
26	NO_2	H	O	45 [>38.3]	40 [>38.3]	ND	ND	>383	>383
27	NH_2	H	O	37 [>43.2]	30 [>43.2]	ND	ND	>432	>432
28	CH_3	H	O	45 [>43.4]	37 [>43.4]	ND	ND	>434	>434
29	C_2H_5	H	O	35 [>41.0]	30 [>41.0]	ND	ND	>410	>410
30	CH_2OH	H	O	0	0	ND	ND	>406	>406
31	CH_2CH_2OH	H	O	25 [>38.4]	28 [>38.4]	ND	ND	>384	>384
32	CH=CHBr	H	O	75−90 [3−15]	53 [30]	ND	ND	>311	>311
33	H			0	0	ND	ND	>465	>465
34	F			20 [>42.9]	0	ND	ND	>429	>429
35	Br			45 [>32.3]	35 [>32.3]	ND	ND	>323	>323
36	H	CH_3	O	27 [>43.4]	0	ND	ND	>434	>434
37	Br	CH_3	O	73 [16.2−32.4]	55 [32.4]	51 [32.4]	30 [>32.4]	>324	>324
38	I	CH_3	O	44 [>28.0]	38 [>28.0]	ND	ND	>280	>280
39	H		O	41 [>46.0]	32 [>46.0]	ND	ND	>460	>460
40	CH_3		O	45 [>43.2]	37 [>43.2]	ND	ND	>432	>432
41	CH_3		S	25 [>40.4]	0	ND	ND	>404	>404
3-TC[g]				96 [0.04−0.21]	88 [2.1−4.4]	45 [>44.0]	30		
Abacavir						80−92 [3.4−16.9]	72 [3.4−16.9]		
GCV[h]				[4.0][i]	20% @ 100 μM[j]	ND	ND	ND	ND

[a] The data are 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 (μM) required to reduce the viral DNA in infected cells to 50% of untreated infected controls. [c] The drug concentration required to reduce the viability of HepG2 cells as determined by MTT assay, by 50% of untreated control after 3 days. [d] (>) sign indicates that 50% inhibition was not reached at the stated (highest) concentration tested. [e] The drug concentration required to reduce the viability of Vero cells as determined by MTT assay, by 50% of untreated control after 3 days. [f] Not determined. [g](−)-3-TC (lamivudine). [h] Ganciclovir. [i] Data taken from Shaw et al. [j] Data taken from Kruining et al.

ethoxy)methyl]uracils (**25**, **30**, **31**) as shown in Scheme 1. The 5,6-disubstituted acyclic pyrimidine nucleosides **37** and **38** were synthesized similarly by direct alkylation of the silylated 6-methyl (**16**), 5-bromo-6-methyl (**17**), and 5-iodo-6-methyl (**18**) bases, respectively (Scheme 1). The site of alkylation of the 5,6-disubstituted acyclonucleosides (**36**−**38**) was established as N-1 by UV spectra determined with solutions at neutral and basic pH, where no or little shift in λ_{max} was observed (experimental data).

Results and Discussion

The anti-HBV activities of the synthesized acyclic pyrimidine nucleosides (**22**−**41**) were evaluated against DHBV in confluent cultures of primary duck hepatocytes obtained from chronically infected Pekin ducks.[48,49] DHBV is a member of the family hepadnaviridae and has a virion structure and genome organization similar to that of human HBV. Thus, DHBV has been used extensively to screen potential drugs to control chronic HBV infection. The anti-HBV activity of the acyclic pyrimidines was also assessed in confluent cultures of the human hepatoma cell line 2.2.15 that chronically produces infectious HBV. 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. To analyze the antiviral effects of the compounds **22**−**41** against the drug-resistant HBV, human hepatoma cell line HepG2 transfected with mutated HBV genome was used. The cell lines contain single mutant HBV (B1 cell line transfected with M204I) or double mutant HBV (D88 cell line transfected with L180M/M204V).[50] Both resistant cell lines have mutations that are clinically relevant. In all of the cell lines used, the antiviral activity was determined by analysis of intracellular viral DNA, using dot blot hybridization. The concentrations required to inhibit 50% of HBV DNA (EC$_{50}$), and 50% cytotoxic concentration (CC$_{50}$) on HepG2 and Vero cells, are shown in Table 1. Lamivudine and abacavir were used in these assays as reference antiviral drugs. Purine acyclic nucleoside ganciclovir (GCV) is also included in Table 1 for comparison of their anti-HBV potency.

Among the acyclic pyrimidine nucleosides investigated, 5-chloro (**25**), 5-(2-bromovinyl) (**32**), and 5-bromo-6-methyl (**37**) analogues exhibited the most promising in vitro activity against DHBV (EC$_{50}$ = 4−20, 3−15, 16.2−32.4 μM, respectively). Interestingly, the anti-DHBV activity exhibited by compounds **25** and **32** was found to be similar to that of corresponding potent acyclic purine nucleoside ganciclovir (EC$_{50}$ = 4 μM), as measured previously in the DHBV infected primary

duck hepatocyte cultures.[51] At a concentration of 10 μg/mL, test compounds **22**, **23**, **24**, **26**−**31**, **33**−**36**, and **38**−**41** exhibited modest or no inhibition of DHBV replication, and the percent inhibition of viral replication was as follows: **22** (0%), **23** (24%), **24** (48%), **26** (45%), **27** (37%), **28** (45%), **29** (35%), **30** (0%), **31** (25%), **33** (0%), **34** (20%), **35** (45%), **36** (27%), **38** (44%), **39** (41%), **40** (45%), and **41** (25%).

Surprisingly, the 5-unsubstituted parent uracil and cytosine derivatives (**22**, **33**) were completely devoid of anti-HBV activity, suggesting that in this series of compounds functionalized substituents at the 5-position of pyrimidine contribute to the antiviral activity. Concerning substitutions by halogens, it appears that the size of the substituent plays an important role, because 5-bromo (**24**) and 5-chloro (**25**) derivatives have higher inhibitory activity than the corresponding 5-fluoro (**23**) analogue. In the case of the 5-bromo compound (**24**), alteration of the 4-position by replacement of the oxygen with methoxy (**35**) did not influence antiviral activity. Similarly, variation of the thymine derivative (**28**) with 6-azathymine (**40**) did not affect activity. At a concentration of 10 μg/mL, compound **28** (45% inhibition) bearing a 5-methyl group had DHBV inhibition similar to that of the 5-bromo (**24**) compound (48% inhibition). Substitution at the 5-position of the uracil base by a longer saturated unsubstituted alkyl group (C_2H_5) or substituted alkyls (CH_2OH, CH_2CH_2OH) reduced the inhibitory activity in contrast to 5-methyl (**28**), 5-bromo (**24**), or 5-chloro (**25**) analogues. Introduction of a double bond at the 5-position of the base increased the inhibitory activity as in compound **32**. These results are in agreement with our previous observations where 5-substituents possessing a double bond provided the most potent anti-HBV activity.[41,52] Similar conclusions were also made by Reimer et al.[53] and Tao et al.[10] who showed that substitution at the 5-position of a pyrimidine nucleoside by an alkyl group containing a double bond increased the inhibitory activity of their triphosphate derivatives against HBV DNA polymerase.

The differences in biological activities for acyclic analogues of pyrimidine nucleosides are likely related to the nature of the substituents at the 5-position of the pyrimidine ring because the conformations with respect to the glycosidic linkage, the 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] chain, are similar in our compounds **22**−**41**.

Comparison of anti-DHBV activity of 5-bromo (**24**) and 5-chloro (**25**) derivatives with related acyclic analogues 1-[(2-hydroxyethoxy)methyl]-5-bromo(or chloro)uracil[47] revealed that chloro and bromo substituents at C-5 positions are important determinants of potent anti-DHBV activity, where the chloro analogue is more potent than the bromo analogue.[47] However, the activity of compounds **24** and **25** containing 1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl] moiety was reduced as compared to their 1-[(2-hydroxyethoxy)methyl] counterparts.[47] Similar observations were also made with compounds containing 5-amino (**27**), 5-hydroxymethyl (**30**), 6-methyl (**36**), or 5-iodo-6-methyl (**38**) substituents.

The antiviral activity of compounds **22**−**41** was also examined against wild-type human hepatitis B virus in 2.2.15 cells. Most of the 5-substituted-[(2-hydroxy-1-(hydroxymethyl)ethoxy)-methyl]uracils active against DHBV retained anti-HBV activity in 2.2.15 cells. The most active compounds **25**, **32**, and **37** exhibited EC_{50} values in the 20−40 μM range that compare favorably to their activity against DHBV. However, it was noteworthy that the anti-HBV activity of compounds **25**, **32**, and **37** in 2.2.15 cells was found to be superior to the corresponding acyclic purine nucleoside ganciclovir (20% inhibition at 100 μM)[38] and only 10−15-fold less than the reference drug lamivudine. This contrasts to their anti-DHBV activity. The differential activity between DHBV and HBV could be attributed to metabolic peculiarities, genomic organization of hepadnavirus (i.e., integrated in 2.2.15 cells and nonintegrated in duck hepatocytes), and/or inherent differences in the human versus duck HBV. Interestingly, like anti-DHBV activity, 5-unsubstituted parent molecules (**22**, **33**) did not show any activity against human HBV in 2.2.15 cells, and similar SARs were observed among various 5-substituents. We further noted that among the compounds **22**−**41** possessing the 1-[(2-hydroxy-1-(hydroxymethyl) ethoxy)methyl] moiety, the antiviral activity against human HBV in 2.2.15 cells was reduced as compared to their 1-[(2-hydroxyethoxy)methyl] counterparts.[47] These results are similar to the observations made for anti-DHBV activity.

Cell-based DNA replication assays were used to measure the antiviral potency of selected compounds **25** and **37** against lamivudine-resistant HBV in a B1 cell line with a single mutant HBV (M204I), and in a D88 cell line with a double mutation (L180M/M204V) (Table 1). The 5-chloro (**25**) and 5-bromo-6-methyl (**37**) derivatives retained activity against single mutant HBV as compared to the wild-type HBV. The anti-HBV activity of **37** was slightly diminished against double-mutant HBV. These results suggest that compounds **25** and **37** are still sensitive to the lamivudine-resistant single and double mutant HBV strains.

It is noteworthy that compounds **25** and **37** were inhibitory against both single and double mutants, and the % inhibition of HBV DNA replication was comparable to that against wild-type HBV (Table 1). Similar results have been reported for the clinical drug adefovir where it exhibited EC_{50} values of 0.58, 0.45−4.9, and 2.2−9.5 μM, respectively, in a wild-type 2.2.15 cell line, and in lamivudine-resistant single and double mutant HBV.[54,55] In contrast, single mutant M204I was >20 times less susceptible to lamivudine than was wild-type HBV. The introduction of the double mutants (L180M/M204V) resulted in HBV that was significantly less sensitive to lamivudine (30% inhibition @10 μg/mL, and EC_{50} was not achieved up to 25 μg/mL). The pattern of activity of lamivudine correlated with clinical investigations where M to V mutation in the YMDD motif of HBV DNA polymerase resulted in a 45-fold decrease in lamivudine susceptibility.[56]

Compounds **22**−**41** were also evaluated for their antiviral activities against West Nile virus, respiratory syncytial virus, SARS-coronavirus, and hepatitis C virus. However, none of these agents exhibited notable inhibition at concentrations up to 100 μg/mL. Compound 1-[(2-hydroxy-1-(hydroxymethyl)-ethoxy)methyl]-5-fluorocytosine (**34**) showed some inhibitory activity against hepatitis C virus (EC_{50} = 132 μM).

The in vitro cytotoxicities of compounds **22**−**41** were assessed in host HepG2 cells as well as Vero cells. No toxicities were observed for these analogues (CC_{50} = >280 to >465 μM (Table 1)).

The fact that compounds **25**, **32**, and **37** show selective anti-HBV activity and low toxicity to host cells suggests that they have higher affinity for the virus-specific DNA polymerase than the host cell enzymes. It is well known that HBV replicates by a multistep mechanism involving the reverse transcription of a pregenomic RNA intermediate, and known anti-HBV agents such as lamivudine and adefovir may act as chain terminators. The distinctive structure of nucleosides **25**, **32**, and **37** may imply that they may not act as chain terminators and their mode of action may be different from that of clinically used agents.

The precise mechanism of action of these compounds remains unclear. However, by analogy with other antiviral nucleosides, the potent anti-HBV activity of these compounds could likely be attributed to their phosphorylation by cellular kinases followed by selective inhibition of HBV DNA polymerase by their triphosphate derivatives acting as substrates and/or inhibitors of HBV DNA polymerase. There is no known HBV-encoded enzyme such as thymidine kinase of the herpes-viruses that could initiate the phosphorylation of anti-HBV nucleosides. In the case of acyclic purine nucleoside, penciclovir, it has been shown that in HBV infected or uninfected host cells, the phosphorylations of penciclovir to its triphosphate form are similar and brought about by cellular enzymes.[57] However, the precise cellular enzymes that may be responsible for phosphorylate acyclic nucleosides are not clear.

Summary

In conclusion, our work demonstrates anti-HBV properties of a new subclass of acyclic pyrimidine nucleoside analogues supporting our strategy for the identification of new anti-HBV agents based on modifications in the sugar moiety of pyrimidine nucleosides. The results obtained here are consistent with our previous observations that 5-substituted acyclic pyrimidine nucleosides are endowed with selective inhibitory activity in vitro not only against wild-type but also for resistant mutant HBV. In this study, compounds possessing 5-chloro, 5-(2-bromovinyl), and 5-bromo-6-methyl substituents on the heterocyclic ring were found to be good inhibitors of HBV replication against DHBV, human HBV, and lamivudine-resistant HBV with a single mutation. The most active compounds exhibit only 10 times lower in vitro anti-HBV activity in 2.2.15 cells than lamivudine, but activity similar to that of the corresponding acyclic guanine derivative ganciclovir against DHBV. Percent inhibition of viral replication by compounds **25** and **37** for resistant mutant HBV was close to that against wild-type HBV in contrast to lamivudine where single and double mutants of HBV were significantly less sensitive than wild-type HBV. Further biochemical studies to elucidate the antiviral mechanism of these acyclic pyrimidine nucleosides and synthesis of additional derivatives are warranted.

The problems of HBV resistance and rebound of viral DNA after drug removal underline the urgent need to design and discover new antiviral strategies and agents. Short-term treatment with lamivudine is usually insufficient to clear the virus. It is possible that resistant mutants may also develop against the investigated new class of compounds. However, new agents are required to be used alone, sequentially, and/or in combination with other drugs to bring more complete virus suppression and prevent or delay drug-resistance problems. There are potential benefits of combination therapy to suppress virus maximally for a longer period and stop the emergence of lamivudine-resistant HBV isolates in patients undergoing monotherapy.

Experimental Section

Melting points were determined with a Buchi capillary apparatus and are uncorrected. ^{1}H NMR spectra were determined for solutions in DMSO-d_6 or CD_3OD on a Bruker AM 300 spectrometer using TMS as an internal standard. The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of D_2O. UV spectra were recorded with a Hewlett-Packard 8453 spectrophotometer. Microanalyses were within ±0.4% of theoretical values for all elements listed, unless otherwise indicated. Silica gel column chromatography was carried out using Merck 7734 silica gel (100−200 μM particle size). Thin-layer chromatography was performed with Machery-Nagel Alugam SiL G/UV silica gel slides (20 μM thickness). 5- and/or 6-substituted uracils (**2**−**21**) were purchased from Sigma-Aldrich Chemical Co.

5-Chloro-1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)methyl]-uracil (25). A mixture of **5** (250 mg, 1.71 mmol), 0.74 g (1.8 mmol) of 2-(bromomethoxy)-1,3-propanediyl-dibenzoate, 0.29 mL (1.79 mmol) of hexamethyldisilazane, 1.2 mL (11.05 mmol) of trimethylchlorosilane, and 1.5 g (4.44 mmol) of potassium nonaflate in 100 mL of dry acetonitrile was refluxed for 5 h at 85 °C. The reaction was brought to room temperature. The solid material was filtered off and washed with dichloromethane (20 mL). The mother liquor and washings were combined and concentrated on rota-vacuo. The residue obtained was redissolved in dichloromethane (300 mL) and washed with water. The organic phase was dried over anhydrous sodium sulfate, filtered, concentrated, and purified with silica gel column chromatography to yield the intermediate 1-[(2-benzoyloxy-(1-benzoyloxymethyl)ethoxy)methyl]uracil that was deprotected using 40% aqueous methylamine (20 mL) at room temperature for 2 h. The product was purified by silica gel column chromatography using MeOH:$CHCl_3$ (1.2:8.8, v/v) as eluent to yield **25** as a solid (200 mg, 46.72%): mp 142−145 °C. ^{1}H NMR (DMSO-d_6): δ 3.40−3.50 (m, 4H, OCH_2CH_2O), 3.55 (m, 1H, CH), 4.62 (m, 2H, OH), 5.20 (s, 2H, NCH_2), 8.18 (s, 1H, H-6), 11.80 (s, 1H, NH).

5-Hydroxymethyl-1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)-methyl]uracil (30). The reaction of **10** (200 mg, 1.41 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.610 g, 1.55 mmol), hexamethyldisilazane (0.23 mL, 1.43 mmol), trimethylchlorosilane (0.95 mL, 8.74 mmol), and potassium nonaflate (1.24 g, 3.66 mmol) in 100 mL of dry acetonitrile at 80 °C for 7 h was followed by debenzolyation using 50 mL of 40% aqueous methylamine at room temperature, using the procedure outlined for the preparation of **22**. Purification of the product by silica gel column chromatography using MeOH:EtoAc (1.5:8.5, v/v) as the eluent yielded **30** as a syrup (80 mg, 23.1%). ^{1}H NMR (DMSO-d_6): δ 3.41−3.58 (m, 5H, OCH_2-CH_2OCH), 4.15 (s, 2H, CH_2OH), 4.60−5.0 (m, 3H, OH), 5.20 (s, 2H, NCH_2), 7.58 (br, 1H, H-6), 11.65 (bs, 1H, NH).

5-(2-Hydroxyethyl)-1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)-methyl]uracil (31). The reaction of **11** (200 mg, 1.28 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.553 g, 1.41 mmol), hexamethyldisilazane (0.26 mL, 1.61 mmol), trimethylchlorosilane (0.96 mL, 8.84 mmol), and potassium nonaflate (1.12 g, 3.31 mmol) in 100 mL of dry acetonitrile at 80 °C for 10 h was followed by debenzolyation using 45 mL of 40% aqueous methylamine at room temperature, using the procedure outlined for the preparation of **22**. Purification of the product by silica gel column chromatography using MeOH:EtOAc (1.5:8.5, v/v) as the eluent yielded **31** as a solid (190 mg, 57.02%): mp 115−118 °C. ^{1}H NMR (DMSO-d_6): δ 2.36 (m, 2H, CH_2), 3.35−3.60 (m, 7H, OCH_2CH_2O, CH, CH_2-OH), 4.55−4.62 (m, 3H, OH), 5.18 (s, 2H, NCH_2), 7.52 (s, 1H, H-6), 11.25 (s, 1H, NH).

1-[(2-Hydroxy-1-(hydroxymethyl)ethoxy)methyl]-6-methyl-uracil (36). The reaction of **16** (200 mg, 1.59 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.69 g, 1.76 mmol), hexamethyldisilazane (0.26 mL, 1.61 mmol), trimethylchlorosilane (1.1 mL, 10.13 mmol), and potassium nonaflate (1.39 g, 4.11 mmol) in 100 mL of dry acetonitrile at 80 °C for 10 h was followed by debenzolyation using 30 mL of 40% aqueous methylamine at room temperature, using the procedure outlined for the preparation of **22**. Purification of the product by silica gel column chromatography using MeOH:EtOAc (1:9, v/v) as the eluent yielded **36** as a white solid (140 mg, 38.35%): mp 140−142 °C. UV λ_{max} (H_2O): 206 nm shoulder (ϵ = 10 200); (0.01 N NaOH) 207 nm (ϵ = 16 600). ^{1}H NMR (DMSO-d_6): δ 2.30 (s, 3H, CH_3), 3.30−3.58 (m, 5H, OCH_2CH_2O, CH), 4.60 (m, 2H, OH), 5.32 (s, 2H, NCH_2), 5.46 (s, 1H, H-5), 11.15 (br s, 1H, NH).

5-Bromo-6-methyl-1-[(2-hydroxy-1-(hydroxymethyl)ethoxy)-methyl]uracil (37). The reaction of **17** (200 mg, 0.98 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.57 g, 1.46 mmol), hexamethyldisilazane (0.2 mL, 1.24 mmol), trimethylchlorosilane (0.73 mL, 6.72 mmol), and potassium nonaflate (0.86 g, 2.53 mmol) in 100 mL of dry acetonitrile at 80 °C for 9 h was followed by debenzolyation using 40 mL of 40% aqueous methylamine at room

temperature, using the procedure outlined for the preparation of **22**. Purification of the product by silica gel column chromatography using MeOH:EtOAc (0.8:9.2, v/v) as the eluent yielded **37** as a white solid (80 mg, 26.54%): mp 145−147 °C. UV λ_{max} (H_2O): 209 nm (ϵ = 7800); (0.01 N NaOH) 213 nm (ϵ = 10 700). ^{1}H NMR (DMSO-d_6): δ 2.58 (s, 3H, CH_3), 3.30−3.46 (m, 4H, OCH_2-CH_2O), 3.56 (m, 1H, CH), 4.38 (m, 1H, OH), 4.60 (m, 1H, OH), 5.42 (s, 2H, NCH_2), 11.75 (s, 1H, NH).

1-[(2-Hydroxy-1-(hydroxymethyl)ethoxy)methyl]-5-iodo-6-methyluracil (38). The reaction of **18** (200 mg, 0.79 mmol) with 2-(bromomethoxy)-1,3-propanediyldibenzoate (0.47 g, 1.2 mmol), hexamethyldisilazane (0.16 mL, 1.0 mmol), trimethylchlorosilane (0.53 mL, 4.88 mmol), and potassium nonaflate (0.7 g, 2.07 mmol) in 100 mL of dry acetonitrile at 80 °C for 9 h was followed by debenzolyation using 35 mL of 40% aqueous methylamine at room temperature, using the procedure outlined for the preparation of **22**. Purification of the product by silica gel column chromatography using MeOH:EtOAc (1.5:8.5, v/v) as the eluent yielded **38** as a solid (80 mg, 28.31%): mp 142−143 °C. UV λ_{max} (H_2O): 215 nm (ϵ = 8500); (0.01 N NaOH) 214 nm (ϵ = 14 800). ^{1}H NMR (DMSO-d_6): δ 2.68 (s, 3H, CH_3), 3.25−3.42 (m, 4H, OCH_2CH_2O), 3.50 (m, 1H, CH), 4.38 (m, 1H, OH), 4.60 (m, 1H, OH), 5.40 (s, 2H, NCH_2), 11.60 (s, 1H, NH).

Biological Evaluations. **In Vitro Antiviral Assay (Duck Hepatitis B Virus, DHBV).** Primary hepatocyte cultures obtained from congenitally infected ducks were used to determine the anti-DHBV activity of test compounds, as reported previously.[45−49] The details of the assay are provided in the Supporting Information.

In Vitro Antiviral Assay (Human Hepatitis B Virus, 2.2.15 Cells). The human HBV transfected 2.2.15 cells were obtained from Dr. M. A. Sells and were used to determine the anti-HBV activity of test compounds, as reported previously.[47,49,58] The details of the assay are provided in the Supporting Information.

In Vitro Antiviral Assay against Lamivudine-Resistant Human Hepatitis B Virus [(B1, M204I) and (D88, L180M/M204V) Cell Lines]. The 3TC-resistant cell lines B1 and D88 were previously constructed by Tyrrell et al. and grown from liquid nitrogen frozen stocks.[50] The D88 cell line contains a double mutation of the HBV genome (rtL180M/M204V), and the B1 cell line features a single mutation (rtM204I). The cell culture, treatment with compounds, and dot blot hybridization procedures were identical to those as described previously for 2.2.15 cells.[47,49,50,58] The details of the assay are provided in the Supporting Information.

Cell Cytotoxicity (MTT Assay). Cytotoxicities of test compounds on human hepatoblastoma cell line (HepG2) and Vero cells were determined using neutral red uptake and MTT assays, respectively, as described earlier.[45−47] The details of the assay are provided in the Supporting Information.

Acknowledgment. We are grateful to the Canadian Institutes of Health Research (CIHR) for an operating grant (MOP-36393) for the financial support of this research. B.A. is thankful to the Alberta Heritage Foundation for Medical Research (AHFMR) for a Medical Scholar Award and EG grant. We also thank the United States National Institutes of Health Antiviral Research Branch, which provided the antiviral tests for West Nile virus, respiratory syncytial virus, and SARS CoV through NIAID contract to Dr. R. W. Sidwell.

Supporting Information Available: The details of the synthesis and in vitro antiviral assays against duck hepatitis B virus, human hepatitis B virus, and lamivudine-resistant human hepatitis B virus; microanalytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Margolis, H. S.; Alter, M. J.; Hadler, S. C. Hepatitis B: Evolving Epidemiology and Implications for Control. *Semin. Liver Dis*. **1991**, *11*, 84−92.
(2) Maddrey, W. C. Hepatitis B-An Important Public Health Issue. *Clin. Lab*. **2001**, *47*, 51−55.
(3) Lok, A. S. Hepatitis B Infection: Pathogenesis and Management. *J. Hepatol*. **2000**, *32 (Suppl. 1)*, 89−97.
(4) Perrillo, R. P.; Schiff, E. R.; Davis, G. L.; Bodenheimer, H. C.; Lindsay, K.; Payne, J.; Dienstag, J. L.; O'Brien, C.; Tamburro, C.; Jacobson, I. M. A Randomized, Control led Trial of Interferon Alfa-2b Alone and After Prednisone Withdrawal for the Treatment of Chronic Hepatitis B. The Hepatitis Interventional Therapy Group. *N. Engl. J. Med.* **1990**, *323*, 295−301.
(5) Wong, D. K. H.; Cheung, A. M.; O'Rourke, K.; Naylor, C. D.; Detsky, A. S.; Heathcote, J. Effect of Alpha-Interferon Treatment in Patients with Hepatitis B e Antigen- Positive Chronic Hepatitis B. A Meta-Analysis. *Ann. Intern. Med.* **1993**, *119*, 312−323.
(6) Torresi, J.; Locarnini, S. Antiviral Chemotherapy for the Treatment of Hepatitis B Virus Infections. *Gastroenterology* **2000**, *118 (Suppl. 2)*, 83−103.
(7) Dunkle, F. M. Frontiers in Drug Development for Viral Hepatitis. ID Weekly Highlights. *Curr. Drugs* **2002**, *Jan.*, 30−32.
(8) Farrell, G. C. Clinical Potential of Emerging New Agents in Hepatitis B. *Drugs* **2000**, *60*, 701−710.
(9) Delaney, W. E.; Locarnini, S., IV; Shaw, T. Resistance of Hepatitis B Virus to Antiviral Drugs: Current Aspects and Directions for Future Investigation. *Antiviral Chem. Chemother*. **2001**, *12*, 1−35.
(10) Tao, P.-Z.; Lofgren, B.; Lake-Bakaar, D.; Johansson, N. G.; Datema, R.; Oberg, B. Inhibition of Human Hepatitis B Virus DNA Polymerase and Duck Hepatitis B Virus DNA Polymerase by Tri-Phosphates of Thymidine Analogues and Pharmacokinetic Properties of the Corresponding Nucleosides. *J. Med. Virol*. **1988**, *26*, 353−362.
(11) Chang, C. N.; Doong, S. L.; Zhou, J. H.; Beach, J. W.; Jeong, L. S.; Chu, C. K.; Tsai, C. H.; Cheng, Y. C.; Liotta, D.; Schinazi, R. Deoxycytidine Deaminase-resistant Stereoisomer is the Active Form of (+/−)-2′,3′-Dideoxy-3′-thiacytidine in the Inhibition of Hepatitis B Virus Replication. *J. Biol. Chem*. **1992**, *267*, 13938−13942.
(12) Doong, S. L.; Tsai, C. H.; Schinazi, R. F.; Liotta, D. C.; Cheng, Y. C. Inhibition of the Replication of Hepatitis B virus In vitro by 2′,3′-Dideoxy-3′-thiacytidine and Related Analogues. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 8495−8499.
(13) Severini, A.; Liu, X. Y.; Wilson, J. S.; Tyrrell, D. L. Mechanism of Inhibition of Duck Hepatitis B Virus Polymerase by (−)-beta-L-2′,3′-Dideoxy-3′-thiacytidine. *Antimicrob. Agents Chemother*. **1995**, *39*, 1430−1435.
(14) Dienstag, J. L.; Schiff, E. R.; Wright, T. L.; Perrillo, R. P.; Hann, H.-W. L.; Goodman, Z.; Crowther, L. B. S.; Condreay, L. D.; Woessner, M. B. S.; Rubin, M.; Brown, N. A.; Brown, N. A. The U.S. Lamivudine Investigator Group. Lamivudine as Initial Treatment for Chronic Hepatitis B in the United States. *N. Engl. J. Med*. **1999**, *341*, 1256−1263.
(15) Lai, C. L.; Chien, R. N.; Leung, N. W.; Chang, T. T.; Guan, R.; Tai, D. I.; Ng, K. Y.; Wu, P. C.; Dent, J. C.; Barber, J.; Stephenson, S. L.; Gray, D. F. A One-year Trial of Lamivudine for Chronic Hepatitis B. Asia Hepatitis Lamivudine Study Group. *N. Engl. J. Med*. **1998**, *339*, 61−68.
(16) Nicoll, A. J.; Colledge, D. L.; Toole, J. J.; Angus, P. W.; Smallwood, R. A.; Locarnini, S. A. Inhibition of Duck Hepatitis B Virus Replication by 9-(2-Phosphonylmethoxyethyl)adenine, an Acyclic Phosphonate Nucleoside Analogue. *Antimicrob. Agents Chemother*. **1998**, *42*, 3130−3135.
(17) Gilson, R. J. C.; Chopra, K.; Murray, L. I. Placebo-controlled Phase I/II Study of Adefovir Dipivoxil in Patients with Chronic Hepatitis B Infection. *Hepatology* **1996**, *24 (Suppl. 1)*, 281A.
(18) Malik, A. H.; Lee, W. M. Chronic Hepatitis B Virus Infection: Treatment Strategies for the Next Millennium. *Ann. Intern. Med*. **2000**, *132*, 723−731.
(19) Hepsera Full Prescribing Information. Gilead Sciences. 09/2002, 1−18.
(20) Zoulim, F. Entecavir: A New Treatment Option for Chronic Hepatitis B. *J. Clin. Virol.*, in press.
(21) De Clercq, E. Perspectives for the Treatment of Hepatitis B Virus Infections. *Int. J. Antimicrob. Agents* **1999**, *12*, 81−95.
(22) (a) Genovesi, E. V.; Lamb, L.; Medina, I.; Taylor, D.; Seifer, M.; Innaimo, S.; Colonno, R. J.; Standring, D. N.; Clark, J. M. Efficacy of the Carbocyclic 2′-Deoxyguanosine Nucleoside BMS-200475 in the Woodchuck Model of Hepatitis B Virus Infection. *Antimicrob. Agents Chemother.* **1998**, *42*, 3209−3217. (b) Levine, S.; Hernaandez, D.; Yamanake, G.; Zhang, S.; Rose, R.; Weinheimer, S.; Colonno, R. J. Efficacies of Entecavir Against Lamivudine-resistant Hepatitis B Virus Replication and Recombinant Polymerases In Vitro. *Antimicrob. Agents Chemother*. **2002**, *46*, 2525−2532.
(23) Tassopoulos, N.; Cianciara, J.; Rizzetto, M. Entecavir is Effective in Treating Patients with Chronic Hepatitis B Who Have Failed Lamivudine Therapy. *Hepatology* **2001**, *34 (Pt 2)*, 340A.

(24) Lai, C. L.; Rosmawati, M.; Lao, J.; Van Vlierberghe, H.; Anderson, F. H.; Thomas, N.; Dehertogh, D. Entecavir is Superior to Lamivudine in Reducing Hepatitis B Virus DNA in Patients with Chronic Hepatitis B Infection. *Gastroenterology* **2002**, *123*, 1831−188.
(25) Nevens, F.; Main, J.; Honkoop, P.; Tyrrell, D. L.; Barber, J.; Sullivan, M. T.; Fevery, J.; DeMan, R. A.; Thomas, H. C. Lamivudine Therapy for Chronic Hepatitis B: A Six Month Randomized Dose-ranging Study. *Gastroenterology* **1997**, *113*, 1258−1263.
(26) Torresi, J.; Locarnini, S. Antiviral Chemotherapy for the Treatment of Hepatitis B Virus Infections. *Gastroenterology* **2000**, *118 (Suppl. 2)*, 83−103.
(27) Delaney, W. E., IV; Edwards, R.; Colledge, D.; Shaw, T.; Torresi, J.; Miller, T. G.; Isom, H. C.; Bock, C. T.; Manns, M. P.; Trautwein, C.; Locarnini, S. Cross-resistance Testing of Antihepadnaviral Compounds Using Novel Recombinant Baculoviruses Which Encode Drug-Resistant Strains of Hepatitis B Virus. *Antimicrob. Agents Chemother*. **2001**, *45*, 1705−1713.
(28) Moraleda, G.; Saputelli, J.; Aldrich, C. E.; Averett, D.; Condreay, L.; Mason, W. S. Lack of Effect of Antiviral Therapy in Nondividing Hepatocyte Cultures on the Closed Circular DNA of Woodchuck Hepatitis Virus. *J. Virol*. **1997**, *71*, 9392−9399.
(29) Addison, W. R.; Walters, K. A.; Wong, W. W.; Wilson, J. S.; Madej, D.; Jewell, L. D.; Tyrrell, D. L. Half-life of the Duck Hepatitis B Virus Covalently Closed Circular DNA Pool in vivo Following Inhibition of Viral Replication. *J. Virol*. **2002**, *76*, 6356−6363.
(30) Zhu, Y.; Yamamoto, T.; Cullen, J.; Saputelli, J.; Aldrich, C. E.; Miller, D. S.; Litwin, S.; Furman, P. A.; Jilbert, A. R.; Mason, W. S. Kinetics of Hepadnavirus Loss from the Liver During Inhibition of Viral DNA Synthesis. *J. Virol*. **2001**, *75*, 311−322.
(31) Liaw, Y. F.; Chien, R. N.; Yeh, C. T.; Tsai, S. L.; Chu, C. M. Acute Exacerbation and Hepatitis B Virus Clearance After Emergence of YMDD Motif Mutation During Lamivudine Therapy. *Hepatology* **1999**, *30*, 567−572.
(32) Allen, M. I.; Deslauriers, M.; Andrews, C. W.; Tipples, G. A.; Walters, K. A.; Tyrrell, D. L.; Brown, N.; Condreay, L. D. Lamivudine Clinical Investigation Group. *Hepatology* **1998**, *27*, 1670−1677.
(33) Fischer, K. P.; Tyrrell, D. L. Generation of Duck Hepatitis B Virus Polymerase Mutants through Site-directed Mutagenesis which Demonstrate Resistance to Lamivudine [(−)-Beta-L-2′,3′-dideoxy-3′-thiacytidine] in vitro. *Antimicrob. Agents Chemother*. **1996**, *40*, 1957−1960.
(34) Melegari, M.; Scaglioni, P. P.; Wands, J. R. Hepatitis B Virus Mutants Associated with 3TC and Famciclovir Administration are Replication Defective. *Hepatology* **1998**, *27*, 628−633.
(35) Das, K.; Xiong, X.; Yang, H.; Westland, C. E.; Gibbs, C. S.; Sarafianos, S. G.; Arnold, E. Molecular Modeling and Biochemical Characterization Reveal the Mechanism of Hepatitis B Virus Polymerase Resistance to Lamivudine (3TC) and Emtricitabine (FTC). *J. Virol*. **2001**, *75*, 4771−4779.
(36) Minuk, G. Y.; German, G. B.; Bernstein, C.; Benarroch, A.; Gauthiar, T.; Sekla, L. A Pilot Study of Steroid Withdrawal Followed by Oral Acyclovir in the Treatment of Chronic Type B Hepatitis. *Clin. Invest. Med*. **1992**, *15*, 506−512.
(37) Luscombe, C.; Pedersen, J.; Uren, E.; Locarnini, S. Long-term Ganciclovir Chemotherapy for Congenital Duck Hepatitis B Virus Infection in vivo. Effect on Intrahepatic-viral DNA, RNA, and Protein Expression. *Hepatology* **1996**, *24*, 766−773.
(38) Kruining, J.; Heijtink, R. A.; Schalm, S. W. Antiviral Agents in Hepatitis B Virus Transfected Cell Lines: Inhibitory and Cytotoxic Effects Related to Time and Treatment. *J. Hepatol*. **1995**, *22*, 263−267.
(39) Chachoua, A.; Dieterich, D.; Krasinski, K.; Greene, J.; Laubenstein, L.; Wernz, J.; Buhles, W.; Koretz, S. 9-(1,3-Dihydroxy-2-propoxymethyl)guanine (Ganciclovir) in the Treatment of Cytomegalovirus Gastrointestinal Disease with the Acquired Immunodeficiency Syndrome. *Ann. Intern. Med*. **1987**, *107*, 133−137.
(40) Beauchamp, L. M.; Serling, B. L.; Kelsey, J. E.; Biron, K. K.; Collins, P.; Selway, J.; Lin, J.-C.; Schaeffer, H. J. Effect of Acyclic Pyrimidines Related to 9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine on Herpes Viruses. *J. Med. Chem*. **1988**, *31*, 144−149.
(41) Ogilvie, K. K.; Hamilton, R. G.; Gillen, M. F.; Radatus, B. K. Uracil Analogues of the Acyclonucleosides 9-[[2-Hydroxy-1-(hydroxymethyl)ethoxy]-methyl]guanine (BIOLF-62). *Can. J. Chem*. **1984**, *62*, 16−21.
(42) Keppler, K.; Kiefer, G.; De Clercq, E. Synthesis and Antviral Activity of Acyclic Derivatives of 5-Ethyl-2′-deoxyuridine. *Arch. Pharm*. **1986**, *319*, 360−365.
(43) Ogilvie, K. K.; Dixit, D. M. Synthesis of 5-Substituted-1-[[2-hydroxy-1-(hydroxymethyl) ethoxy]methyl]cytosines. *Nucleosides Nucleotides* **1983**, *2*, 147−154.
(44) (a) Martin, J. C.; Jeffrey, G. A.; McGee, D. P.; Tippie, M. A.; Smee, D. F.; Matthews, T. R.; Verheyden, J. P. Acyclic Analogues of 2′-Deoxynucleosides Related to 9-[(1,3-Dihydroxy-2-propoxy)methyl]-guanine as Potential Antiviral Agents. *J. Med. Chem*. **1985**, *28*, 358−62. (b) Chein, H. H.; Chen, Y. L.; Tzeng, C. C. Synthetic Studies on the Acyclic Nucleosides of 5-Substituted 6-Aza Uracils. *Nucleosides Nucleotides* **1991**, *10*, 1391−1406.
(45) Kumar, R.; Sharma, N.; Nath, M.; Saffran, H. A.; Tyrrell, D. L. J. Synthesis and Antiviral Activity of Novel Acyclic Nucleoside Analogues of 5-(1-Azido-2-haloethyl)uracils. *J. Med. Chem.* **2001**, *44*, 4225−4229.
(46) Kumar, R.; Nath, M.; Tyrrell, D. J. Design and Synthesis of Novel 5-Substituted Acyclic Pyrimidine Nucleosides as Potent and Selective Inhibitors of Hepatitis B Virus. *J. Med. Chem*. **2002**, *45*, 2032−2040.
(47) Semaine, W.; Johar, M.; Tyrrell, D. L. J.; Kumar, R.; Agrawal, B. Inhibitors of Hepatitis B Virus (HBV) Replication by Pyrimidines Bearing Acyclic Moiety: Effect on Wild-type and Mutant HBV. *J. Med. Chem*. **2006**, *49*, 2049−2054.
(48) Lee, B.; Luo, W.; Suzuki, S.; Robins, M. J.; Tyrrell, D. L. J. In vitro and in vivo Comparison of the Abilities of Purine and Pyrimidine 2′,3′-Dideoxynucleosides to Inhibit Duck Hepadnavirus. *Antimicrob. Agents Chemother*. **1989**, *33*, 336−339.
(49) Tuttleman, J. S.; Pugh, J. C.; Summers, J. W. In vitro Experimental Infection of Primary Duck Hepatocyte Cultures with Duck Hepatitis B Virus. *J. Virol.* **1986**, *58*, 17−25.
(50) Walters, K. A.; Tipples, G. A.; Allen, M. I.; Condreay, L. D.; Addison, W. R.; Tyrrell, L. Generation of Stable Cell Lines Expressing Lamivudine-Resistant Hepatitis B Virus for Antiviral-Compound Screening. *Antimicrob. Agents Chemother*. **2003**, *47*, 1936−1942.
(51) Shaw, T.; Amor, P.; Civitico, G.; Boyd, M.; Locarnini, S. In Vitro Antiviral Activity of Penciclovir, A Novel Purine Nucleoside, Against Duck Hepatitis B Virus. *Antimicrob. Agents Chemother*. **1994**, *38*, 719−723.
(52) Kumar, R.; Tyrrell, D. J. Novel 5-Vinyl Pyrimidine Nucleosides with Potent anti-Hepatitis B Virus Activity. *Bioorg. Med. Chem. Lett*. **2001**, *11*, 2917−2920.
(53) Reimer, K.; Matthes, E.; Janta-Lipinski, M. V.; Meisel, H. Inhibition of Hepatitis B Virus DNA Polymerase by Thymidine Phosphate Analogues in Vitro. *Antiviral Chem. Chemother*. **1991**, *2*, 249−253.
(54) Ono, S. K.; Kato, N.; Shiratori, Y.; Kato, J.; Goto, T.; Schinazi, R. F.; Carrilho, F. J.; Omata, M. The polymerase L528M Mutation Cooperates with Nucleotide Binding-site Mutations, Increasing Hepatitis B Virus Replication and Drug Resistance. *J. Clin. Invest.* **2001**, *107*, 449−455.
(55) Perrillo, R.; Schiff, E.; Yoshida, E.; Statler, A.; Hirsch, K.; Wright, T.; Gutfreund, K.; Lamy, P.; Murray, A. Adefovir Dipivoxil for the Treatment of Lamivudine-resistant Hepatitis B Mutants. *Hepatology* **2000**, *32*, 129−134.
(56) Bartholomew, M. M.; Jansen, R. W.; Jeffers, L. J.; Reddy, K. R.; Johnson, L. C.; Bunzendahl, H.; Condreay, L. D.; Tzakis, A. G.; Schiff, E. R.; Brown, N. A. Hepatitis-B-Virus Resistance to Lamivudine Given for Recurrent Infection after Orthotopic Liver Transplantation. *Lancet* **1997**, *349*, 20−22.
(57) Korba, B. E.; Boyd, M. R. Penciclovir is a Selective Inhibitor of Hepatitis B Virus Replication in Cultured Human Hepatoblastoma Cells. *Antimicrob. Agents Chemother.* **1996**, *40*, 1282−1284.
(58) Sells, M. A.; Chen, M. L.; Acs, M. L. Production of Hepatitis B Virus Particles in Hep G2 Cells Transfected with Cloned Hepatitis B Virus DNA. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 1005−1009.

JM060102L