Inhibition of Hepatitis B Virus (HBV) Replication by Pyrimidines Bearing an Acyclic Moiety: Effect on Wild-Type and Mutant HBV

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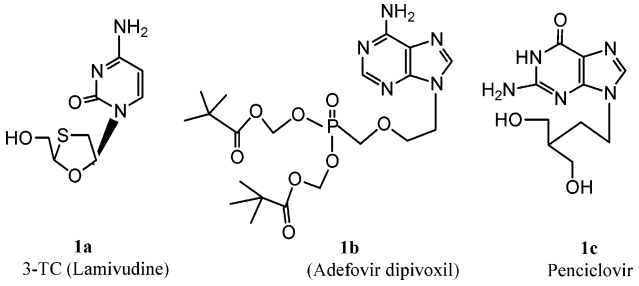
Chronic hepatitis B virus (HBV) infection remains a major health problem worldwide. The main clinical limitation of a current antiviral drug for HBV, lamivudine, is the emergence of drug-resistant viral strains upon prolonged therapy. A group of 5-, 6-, or 5,6-substituted acyclic pyrimidine nucleosides with a 1-[(2-hydroxyethoxy)methyl] moiety were synthesized and evaluated for antiviral activities. The target compounds were prepared by the reaction of silylated uracils possessing a variety of substituents at the C-5 or C-6 positions or both with 1,3-dioxolane in the presence of potassium iodide and chlorotrimethylsilane by a convenient and single-step synthesis. Among the compounds tested, 5-chloro and 5-bromo analogues possessing an acyclic glycosyl moiety were the most effective and selective antiviral agents in the in vitro assays against wild-type duck HBV (EC_{50} = 0.4−2.2 and 3.7−18.5 μM, respectively) and human HBV-containing 2.2.15 cells (EC_{50} = 4.5−45.4 and 18.5−37.7 μM, respectively). These compounds were also found to retain sensitivity against lamivudine-resistant HBV containing a single mutation (M204I) and double mutations (L180M/M204V). The compounds investigated did not show cytotoxicity to host HepG2 and Vero cells, up to the highest concentration tested. The results presented here confirm and accentuate the potential of acyclic pyrimidine nucleosides as anti-HBV agents and extend our previous observations. We herein report the capability of acyclic pyrimidine nucleosides to inhibit the replication of both wild-type and drug-resistant mutant HBV.

Introduction

Hepatitis B virus (HBV) infection is one of the leading causes of death due to infectious diseases worldwide. There are approximately 400 million people with chronic HBV infection, with an annual global death toll of 1.2 million per year.[1,2] Chronic HBV infected people are 10 times more numerous than HIV (human immunodeficiency virus) patients. An HBV infected mother can pass the infection to her nonvaccinated infant at the time of birth.[3,4] Neonatal exposure to HBV results in chronic HBV infection in 90% of the cases, whereas adults exposed to HBV result in chronic infection in only 10% of the cases.[3] In the chronic carriers, 25−40% of the people develop liver cirrhosis and hepatocellular carcinoma leading to significant mortality.

Until recently, the major therapeutic option for HBV carriers was α-interferon. However, its use is limited because the success rate is low and serious side effects are observed.[5,6] Lamivudine [(−)-β-L-2′,3′-dideoxy-3′-thiacytidine, (−)3TC, **1a**, Figure 1] has been clinically used for the treatment of HBV infection for a few years. It is orally effective and well tolerated and shows marked inhibition of HBV DNA levels, loss of HBeAg (hepatitis B e antigen), seroconversion (defined as HBeAg-negative, antibodies to HBeAg-positive, and HBV-DNA-negative) and clinical improvements in the liver inflammation and histology in the majority of patients.[7,8] Recently, adefovir dipivoxyl [9-[2-[bis[(pivaloyloxy)methoxy]phosphinyl]methoxy]-ethyl]-adenine, **1b**] has also been approved as another antiviral agent for the treatment of chronic HBV infection. Adefovir was



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

Figure 1.

demonstrated to be well-tolerated, to reduce serum HBV DNA levels significantly, and to lead to seroconversion in 20−27% of the patients treated for 12 weeks at a daily dose of 30 mg/day or greater.[1,9] 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.[10] In clinical trials, after the discontinuation of adefovir, 25% of patients had exacerbations of hepatitis (alanine aminotransferase (ALT) elevation of 10-times the upper limit of normal levels or greater) and there were even fatalities.[10]

Treatment with lamivudine or adefovir in HBV infected patients leads to quantitative decrease in HBV replication. However, after cessation of treatment, virus DNA levels rebound to baseline pretreatment or even higher levels.[7,11,12] Therapy with lamivudine and adefovir inhibits cytoplasmic HBV replication but has no direct effect on HBV covalently closed circular DNA (cccDNA).[11,12] However, continuous and complete suppression of HBV DNA replication may deplete cccDNA.[13] Short-term therapy with HBV DNA synthesis inhibitors cannot deplete the pool of cccDNA, and this could be one reason for rapid rebound of viral replication after cessation of therapy.[14] Continuous long-

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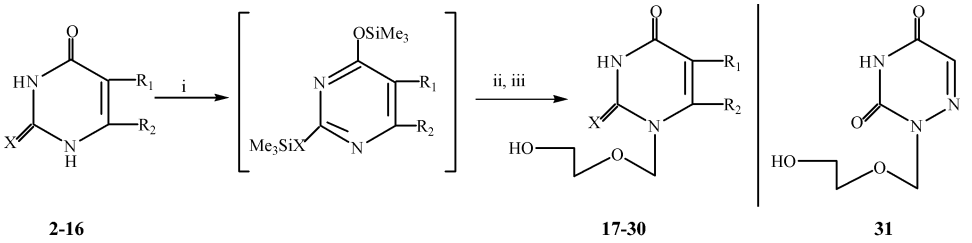
Scheme 1[a]



2-16 **17-30** **31**

2 = R_1 = Br, R_2 = H, X = O
3 = R_1 = Cl, R_2 = H, X = O
4 = R_1 = I, R_2 = H, X = O
5 = R_1 = NO_2, R_2 = H, X = O
6 = R_1 = NH_2, R_2 = H, X = O
7 = R_1 = C_2H_5, R_2 = H, X = S
8 = R_1 = H, R_2 = H, X = S
9 = R_1 = CF_3, R_2 = H, X = O
10 = R_1 = CH_2OH, R_2 = H, X = O
11 = R_1 = $COOC_2H_5$, R_2 = H, X = S
12 = R_1 = H, R_2 = CH_3, X = O
13 = R_1 = H, R_2 = $COOCH_3$, X = O
14 = R_1 = I, R_2 = CH_3, X = O
15 = R_1 = NO_2, R_2 = CH_3, X = O
16 = 6-Azauracil

17 = R_1 = Br, R_2 = H, X = O
18 = R_1 = Cl, R_2 = H, X = O
19 = R_1 = I, R_2 = H, X = O
20 = R_1 = NO_2, R_2 = H, X = O
21 = R_1 = NH_2, R_2 = H, X = O
22 = R_1 = C_2H_5, R_2 = H, X = S
23 = R_1 = H, R_2 = H, X = S
24 = R_1 = CF_3, R_2 = H, X = O
25 = R_1 = CH_2OH, R_2 = H, X = O
26 = R_1 = $COOC_2H_5$, R_2 = H, X = S
27 = R_1 = H, R_2 = CH_3, X = O
28 = R_1 = H, R_2 = $COOCH_3$, X = O
29 = R_1 = I, R_2 = CH_3, X = O
30 = R_1 = NO_2, R_2 = CH_3, X = O

[a] Reagents: (i) Bis(trimethylsilyl)acetamide, dry acetonitrile; (ii) 1,3-dioxolane, potassium iodide, trimethyl chlorosilane, 25 °C; (iii) quenched with MeOH and neutralized with $NaHCO_3$.

term therapy with an array of potent nontoxic individual anti-HBV agents or a combination of anti-HBV agents may lead to continuous inhibition of viral DNA synthesis resulting eventually in the depletion of the cccDNA pool. Depletion of cccDNA can be achieved, as has been shown with penciclovir (PCV, **1c**) in duck HBV (DHBV)-infected hepatocytes.[15]

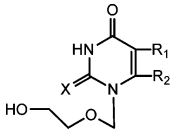
A significant clinical problem with antiviral therapy in chronic HBV and liver transplant patients has been the emergence of resistance to lamivudine.[13,16−22] Genotypic resistance emerges in 14−32% of patients within the first 12 months of lamivudine therapy, increasing to 40% within 2 years of treatment and 57% by the 3rd year.[8,23−25] Initially, resistance to lamivudine was identified with mutations in a conserved functional epitope of HBV DNA polymerase in the YMDD motif in lamivudine-treated patients.[18] Subsequently, it was found that resistance to lamivudine occurs most often in the conserved YMDD motif of the nucleotide-binding site of the viral polymerase.[20,26−28] Single nucleotide changes at codon 204 of the reverse transcriptase domain of the polymerase result in the substitution of either valine or isoleucine for methionine (M204V or M204I). The valine substitution and occasionally the isoleucine substitution are accompanied by an additional upstream mutation at codon 180, where a methionine is substituted for a leucine (L180M).[26] Adefovir, lobucavir, and penciclovir (purine nucleotide and nucleoside analogues) have been shown to be active against lamivudine-resistant HBV.[29,30] Therefore, nucleoside analogues that have sugar ring systems that are modified or acyclic may retain activity against lamivudine-resistant mutants.[29] This permits the possibility that lamivudine-resistant HBV could be treated with other nucleoside agents singly or in combination. It is therefore important to continue research to develop new antiviral strategies to better combat wild-type and mutant HBV infections.

Acyclic pyrimidine nucleosides have previously been shown to have antiviral activity against herpes simplex virus.[31] We have recently reported pyrimidine nucleosides with acyclic structures as selective inhibitors of DHBV.[32−34] This new class of molecules displayed strong inhibition of HBV replication in primary duck hepatocytes infected with DHBV in vitro.[32−34] Given the novelty of their structures and in effort to further explore the structure−activity relationships (SARs) in this series of promising antivirals, we now report synthesis and anti-HBV activity of various 5-, 6- or 5,6-substituted analogues of 1-[(2-hydroxyethoxy)methyl]uracils (**17**−**31**). In these studies, we note that 5-halo analogues **17** and **18** in particular display potent and selective anti-HBV activity against duck HBV, wild-type human HBV, and lamivudine-resistant HBV in vitro.

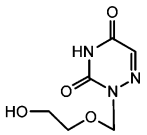
Chemistry

The target compounds 5-, 6-, or 5,6-substituted 1-[(2-hydroxyethoxy)methyl]uracils (**17**−**31**) were achieved by a simple and convenient one-step synthesis using iodomethyl [(trimethylsilyl)oxy]ethyl ether, prepared in situ from 1,3-dioxolane and trimethyl chlorosilane, instead of acetoxyethyl acetoxymethyl ether as alkylating reagent.[31,35,36] By this method, steps involving synthesis of the acyclic chain (2-acetoxyethyl acetoxymethyl ether) and deprotection of the acetyl group were eliminated. Thus uracils **2**−**16** silylated with bis(trimethylsilyl)-acetamide in dry acetonitrile were reacted with trimethylchlorosilane, potassium iodide, and 1,3-dioxolane at room temperature for 16−24 h to yield the desired compounds **17**−**31** (Scheme 1). In ^{1}H NMR, the chemical shifts of NCH_2 protons in 6-substituted and 5,6-disubstituted derivatives (**27**−**30**) were observed at δ 5.12−5.36, a similar region as those observed for 5-substituted derivatives (**17**−**21**, **24**, and **25**, δ 5.07−5.28), providing evidence for the of N-1 alkylation in compounds **27**−**30**. It is expected that N-3 alkylated product would have resulted in the NCH_2 protons being at much lower field than those in the N-1 alkylated product due to the electron-withdrawing properties of the two adjacent carbonyl functionalities. Further, the site of alkylation of the acyclonucleosides **27**−**30** 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).

Table 1. In Vitro Activity against Hepatitis B Virus and Toxicity of 5-, 6- or 5,6-Substituted Analogues of 1-[(2-Hydroxyethoxy)methyl]uracils



17-30



31

				% inhibition at10 μg/mL[a]		[EC$_{50}$ (μM)] [b,h]		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,d]	Vero cells[d,e]
17	Br	H	O	89 [3.7−18.5]	60−73 [18.5−37.7]	67 [18.5−37.7]	73 [3.7−37.7]	>377	>377
18	Cl	H	O	94 [0.4−2.2]	82 [4.5−45.4]	69 [4.5−45.4]	70 [4.5−45.4]	>454	>454
19	I	H	O	50 [32.0]	*f*	*f*	*f*	>320	>320
20	NO_2	H	O	53 [43.2]	54 [43.2]	30	0	>432	>432
21	NH_2	H	O	65 [5.0]	50 [50.0]	30	30	>497	>497
22	C_2H_5	H	S	45 [>47.8]	40 [>47.8]	20	40 [>47.8]	>478	>478
23	H	H	S	46 [>49.5]	42 [>49.5]	30	0	>495	>495
24	CF_3	H	O	76 [3.9−39.3]	55−65 [19.6−39.3]	53 [39.3]	50 [39.3]	>393	>393
25	CH_2OH	H	O	55 [47.6]	50−73 [23.8−47.6]	0	30	>476	>476
26	$COOC_2H_5$	H	S	53 [36.4]	50−64 [18.2−36.4]	20	0	>364	>364
27	H	CH_3	O	60 [25]	55−72 [25−50]	30	0	>500	>500
28	H	$COOCH_3$	O	56 [40.9]	60 [40.9]	0	0	>409	>409
29	I	CH_3	O	51 [30.6]	53 [30.6]	30	25	>306	>306
30	NO_2	CH_3	O	68 [20.4−40.8]	60−80 [20.4−40.8]	25	20	>408	>408
31				25 [>53]	25 [>53]	*f*	*f*	>534	>534
3-TC[g]				96 [0.04−0.2]	88 [2.1−4.4]	45 [>44]	30		
abacavir						80−90 [3.4−16.9]	72 [3.4−16.9]		

[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] The > 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] (−)-β-L-2′,3′-dideoxy-3′-thiacytidine. [h] Percent inhibition was calculated by using the formula (untreated positive control − treated test sample) × 100/untreated positive control. Tests were repeated 2−3 times, and the data for each test compound were compared with a positive and negative control performed at the same time under identical conditions. For the compounds where the EC$_{50}$ obtained from three experiments was within 10% standard deviation, average values are shown, otherwise a range of EC$_{50}$ values are shown.

Results and Discussion

To assess the antiviral effect of 5-, 6-, or 5,6-substituted analogues of 1-[(2-hydroxyethoxy)methyl]uracils (**17**−**31**) on wild-type HBV replication in vitro, primary hepatocytes from ducks congenitally infected with duck HBV were used. These cells chronically produce DHBV DNA, and therefore antiviral activity was determined by analysis of intracellular viral DNA using dot-blot hybridization. Stable HBV-producing human hepatoblastoma cell line 2.2.15, which carries HBV DNA stably integrated into the genome of HepG2 cells, was used to determine the antiviral activity of compounds **17**−**31** against human hepatitis B virus in vitro. To analyze the antiviral effect of the compounds **17**−**31** against drug-resistant HBV, human hepatoma cell line HepG2 transfected with the 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).[37] Both resistant cell lines have mutations that are clinically relevant. The concentrations required to inhibit 50% of HBV DNA (EC$_{50}$) and the 50% cytotoxic concentration (CC$_{50}$) on HepG2 and Vero cells are shown in Table 1. Lamivudine (3-TC) and abacavir were used in these assays as reference antiviral drugs.

Among the acyclic nucleosides studied, 5-iodo (**19**), 5-nitro (**20**), 5-hydroxymethyl (**25**), and 5-carboxyethyl (**26**) analogues inhibited the replication of DHBV effectively with EC$_{50}$ values of 32−47.6 μM. The corresponding 5-bromo (**17**), 5-chloro (**18**), 5-amino (**21**), and 5-trifluoromethyl (**24**) analogues were active and inhibited 50% DHBV replication at 3.7−18.5, 0.4−2.2, 5.0, and 3.9−39.3 μM, respectively. In the 6-substituted and 5,6-disubstituted series of compounds, 6-methyl (**27**), 6-carbomethoxy (**28**), 5-iodo-6-methyl (**29**), and 5-nitro-6-methyl (**30**) derivatives also exhibited anti-DHBV activity, where they were approximately equiactive (EC$_{50}$ = 20.4−40.9 μM). In the DHBV studies, 5-chloro derivative **18** (EC$_{50}$ = 0.4−2.2 μM) emerged as the most active analogue. The bromo derivative **17** was approximately 9-fold less active (EC$_{50}$ = 3.7−18.5 μM) than **18**. In contrast compound **19** with a 5-iodo substituent was less inhibitory to DHBV replication (EC$_{50}$ = 32 μM) than either **17** or **18**. These results suggest that halogen substituents at the 5-position are determinants of anti-DHBV activity in this series of compounds. The anti-DHBV activity exhibited by the most active compound **18** was 10-times less potent than that of reference drug 3-TC (EC$_{50}$ = 0.04−0.2 μM).

The activity of compounds **17**−**31** against wild-type HBV was also examined against human hepatitis B virus in 2.2.15 cells. Most of the compounds active against DHBV retained anti-HBV activity in 2.2.15 cells. The most active compound **18** exhibited an EC$_{50}$ value of 4.5−45.4 μM in 2.2.15 cells that compares favorably to that of reference drug 3-TC (EC$_{50}$ = 2.1−4.4 μM). We note that anti-HBV activity for compounds **17**, **18**, and **21** in 2.2.15 cells was diminished 2−10-fold and 10-fold, whereas for compounds **25** and **26**, it was improved approximately by 2-fold as compared to DHBV. The reasons for the observed differences between the two cell systems may be related to metabolic peculiarities, genomic organization of hepadnavirus (i.e., integrated in 2.2.15 cells and nonintegrated in duck hepatocytes), or inherent differences in the human vs duck HBV.

1-[(2-Hydroxyethoxy)methyl]-5-azauracil (**31**) possessed significantly reduced anti-HBV activity against wild-type duck HBV and human HBV (25% inhibition at 10 μg/mL), suggesting that replacement of carbon with a nitrogen atom at the 6-position of the uracil base is detrimental to antiviral activity.

Cell-based DNA replication assays were used to measure the antiviral activity of compounds **17**−**31** against lamivudine-resistant HBV. In the B1 cell line with single-mutant HBV, compounds **20**−**23**, **26**, **27**, **29**, and **30** showed moderate activity (20−40%) in HBV replication at 10 μg/mL, whereas 5-chloro (**18**), 5-bromo (**17**), and 5-trifluoromethyl (**24**) analogues were notably inhibitory with EC_{50} values of 4.5−45.4, 18.5−37.7, and 39.3 μM, respectively. In the D88 cell line with double mutations (L180M/M204V), compounds **21**, **22**, **25**, **29**, and **30** exhibited moderate activity (20−40%) in HBV replication at the concentration of 10 μg/mL, whereas compound **24** exhibited an EC_{50} of 39.3 μM. Encouragingly, acyclic derivatives **17** and **18** showed better response against lamivudine-resistant HBV with double mutations with EC_{50} values of 3.7−45.4 μM suggesting that L180M/M204V changes do not confer significant cross-resistance to them. The anti-HBV activity exhibited by **17** and **18** compares favorably to reference compound abacavir (EC_{50} = 3.4−16.9 μM) used in these assays. Abacavir has been reported to be active against lamivudine-resistant HBV where no significant shift in the EC_{50} was observed for virus expressing either the M204I mutation or the L180M/L204V mutations compared to that for the wild-type virus.[37] It is interesting to observe that compounds **17** and **18** were effective against both single and double mutants, and the EC_{50} values of these were approximately similar to that against wild-type HBV (Table 1). Similar results have been reported for the clinical drug adefovir where it exhibits EC_{50} values of 0.58, 0.45−4.9, and 2.2−9.5 μM, respectively, in the wild-type HepG2 2.2.15 cell line and lamivudine-resistant single- and double-mutant HBV.[38,39] In contrast, the single mutant M204I was >20 times less susceptible to 3-TC than was wild-type HBV. The introduction of the double mutations (L180M/M204V) resulted in HBV that was significantly less sensitive to 3-TC (30% inhibition at 10 μg/mL, and EC_{50} was not achieved up to 25 μg/mL). The pattern of activity of lamivudine correlated with clinical in vitro investigations where the methionine to valine mutation in the YMDD motif of HBV DNA polymerase resulted in a 45-fold decrease in lamivudine susceptibility.[18]

The compounds **17**−**31** 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. 1-[(2-Hydroxyethoxy)methyl]-5-nitrouracil (**20**) showed some inhibitory activity against West Nile virus (EC_{50} > 134 μM). The compounds **17**−**31** exhibited no in vitro cytotoxicity against host HepG2 and Vero cells (CC_{50} >306−534 μM) (Table 1).

Summary

Infection with hepatitis B virus is a medical problem of global proportions. We describe 5-, 6-, or 5,6-substituted analogues of 1-[(2-hydroxyethoxy)methyl]uracil as HBV-specific acyclic pyrimidine nucleosides in vitro. While compounds **17**, **18**, **21**, **22**, **24**, **29**, and **30** showed inhibition against wild-type duck HBV, human HBV (2.2.15), and lamivudine-resistant single and double mutants of HBV, the 5-chloro (**17**) and 5-bromo (**18**) analogues containing a modified glycosyl portion are most effective against all wild-type and mutant HBV tested. The observed antiviral activities of 5-, 6-, or 5,6-substituted acyclic pyrimidine nucleosides against wild-type and resistant HBV mutants is important for identification of new and effective anti-HBV agents with different resistance patterns or mechanisms of action. Further biological and structure−activity relationship studies of this new class of anti-HBV agents are ongoing in our laboratories.

Experimental Section

Melting points were determined with a Buchi capillary apparatus and are uncorrected. ^{1}H NMR spectra were determined for solutions in Me_2SO-d_6 on a Bruker AM 300 spectrometer using Me_4Si as an internal standard. The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of the 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). The 5-, 6-, or 5,6-substituted uracils (**2**−**16**) were purchased from Sigma-Aldrich Chemical Co.

1-[(2-Hydroxyethoxy)methyl]-5-bromouracil (17). To a suspension of **2** (3 g, 15.7 mmol) in 40 mL of dry acetonitrile, 9.0 mL (44.2 mmol) of bis(trimethylsilyl)acetamide was added, and the mixture was stirred at room temperature till a clear solution was obtained. To this solution, 1.2 mL (16.2 mmol) of 1,3-dioxolane, 2.6 g (15.6 mmol) of potassium iodide (KI), and 2.4 mL (22.09 mmol) of chlorotrimethylsilane were added. The reaction mixture was stirred at room temperature for 16 h at which time TLC (EtOAc/MeOH, 9.5:0.5, v/v) indicated that the reaction was completed. The reaction medium was quenched with methanol and neutralized with 6.0 g of sodium bicarbonate. The solid obtained was purified by silica gel column chromatography using EtOAc/MeOH (99:1, v/v) to yield **17** as a solid (3.85 g, 92.5%): mp 150−152 °C (lit mp 147−148 °C);[31] ^{1}HNMR (DMSO-d_6) δ 3.55 (m, 4H, OCH_2CH_2O), 4.65 (br s, 1H, OH), 5.14 (s, 2H, NCH_2), 8.28 (s, 1H, H-6). Anal. ($C_7H_9BrN_2O_4$) C, H, N.

The above procedure used for the synthesis of **17** was applied for the preparation of compounds **18**−**31** with variations as described in Supporting Information.

In Vitro Antiviral Assay for 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.[32,34,40,41]

In Vitro Antiviral Assay for Human Hepatitis B Virus Using 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.[41,42]

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 out of liquid nitrogen frozen stocks.[37] 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 similar to a procedure with 2.2.15 cells as described previously.[37,41,42]

Cell Cytotoxicity (MTT Assay). Cytotoxicities of test compounds on HepG2 and Vero cells were determined using neutral red uptake and MTT assays, respectively, as described earlier.[32,34]

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Nile virus, respiratory syncytial virus, SARS coronavirus, and hepatitis C virus through NIAID contract to Dr. R.W. Sidwell.

Supporting Information Available: The details of synthesis of compounds **18**−**31** and their spectral and elemental analyses.[43] This material is available free of charge via the Internet at http://pubs.acs.org.

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