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Synthesis and in vitro antiviral activities of 3′-fluoro (or chloro) and 2′,3′-difluoro 2′,3′-dideoxynucleoside analogs against hepatitis B and C viruses

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ABSTRACT

Chronic infections with hepatitis B virus (HBV) and hepatitis C virus (HCV) lead to serious liver diseases worldwide. Co-infection with HBV and HCV is very common and is associated with increased risk of liver pathogenesis, liver cancer, and liver failure. Several 5-substituted 3′-fluoro (or chloro) (**1–4**, **6, 7, 17–19**) and 2′,3′-difluoro 2′,3′-dideoxynucleosides (**15 and 16**) were synthesized and evaluated for in vitro antiviral activities against duck hepatitis B virus (DHBV), human hepatitis B virus, and hepatitis C virus. Of these compounds **4, 7, 17**, and **19** demonstrated moderate anti-HBV activity, and **2**, **4, 7, 8**, and **19** were weak inhibitors of HCV. Although 5-iodo derivative (**7**) was most inhibitory against HCV, it exhibited a reduction in cellular RNA levels in Huh-7 cells. The 5-hydroxymethyl-3′-fluoro-2′,3′-dideoxyuridine (**4**) and 1-(3-chloro-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)-5-fluorouracil (**19**) provided the most inhibition of both viruses without cytotoxicity.

1. Introduction

Hepatitis B and C viruses (HBV and HCV) belong to hepadnaviridae and flaviviridae families, respectively. The infection with these two hepatitis viruses is the major cause of serious liver diseases such as hepatitis, liver failure, cirrhosis, and hepatocellular carcinoma worldwide.[1–3]

Approximately 2 billion people have been infected with HBV and ~400 million people worldwide are chronically infected with HBV (chronic carriers of HBV).[4] Up to 1.2 million deaths occur annually due to HBV.[5] The HBV genome consists of a partly double stranded DNA, but goes through reverse transcription of an RNA intermediate to complete genome replication. Current vaccine for HBV, although useful for preventing new infections, is not useful for the chronic carriers. The antiviral therapy with approved nucleoside/nucleotide agents has limitations due to emergence of resistance, side effects, and/or viral rebound after the cessation of therapy.[6]

Worldwide, approximately 170 million people are chronically infected with HCV.[7] Unlike HBV infection where most of the adults acquiring HBV develop acute infection and clear the virus, 70–85% of the people acquiring HCV develop chronic infection and later liver diseases. HCV contains a single stranded RNA genome and goes through replication via RNA dependent RNA polymerase.[8] There is no vaccine available for HCV, and the current standard of therapy peg-IFN-α and ribavirin is expensive, limited in efficacy and associated with serious side effects.[9,10] Further, this therapy is only effective in <50% of the people with genotype 1a, the most prevalent genotype in North America.[11]

People co-infected with both HBV and HCV demonstrate a faster progression to liver fibrosis, more severe liver disease, increased risk of developing hepatocellular carcinoma, and markedly increased risk of liver related death and overall mortality.[12,13] Currently, there is no standard of care treatment for HBV/HCV co-infected patients. IFN-α/ribavirin treatment showed lower response rate in co-infected patients compared to mono-infected HCV patients in a clinical trial.[14] Due to the limited treatment options available currently and their limited efficacy, new antiviral agents are urgently required for the treatment of both HBV and HCV.

Due to common mode of parenteral transmission, HIV-infected individuals also have a high prevalence of multiple hepatitis co-infection (HBV and HCV).[15] However, limited studies are available on the virological aspects, clinical impact and therapeutic aspects of HIV-infected people co-infected with HBV and HCV. In HIV positive patients, the immunodeficiency may lead to increase in both HCV and HBV replication with a more aggressive liver disease, and the use of anti-retroviral agents (some of which also inhibit HBV replication), may influence the dynamics of HCV/HBV co-infection.[16,17]

In earlier studies, 3′-fluorinated pyrimidine nucleosides have been investigated as anti-HIV agents.[18] Of these, 3′-fluoro-2′,3′-dideoxythymidine (FddThd) and 3′-fluoro-2′,3′-dideoxy-5-chloro-

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uridine (FddClUrd) emerged as the most active inhibitors of HIV-1 replication.[18] 3′-Fluorinated nucleosides have also been explored as anti-HBV agents where 2′,3′-dideoxyguanosine (FLG) has shown promise against duck HBV (DHBV) and human HBV (HBV) and has undergone clinical trials.[19] FddThd and FddClUrd were also found to be strong inhibitors of HBV in vitro using the HepG2 2.2.15 cells. FddThd was effective against DHBV in vivo.[20] Inspite of the potent anti-HBV activity shown by FddThd, its long-term use as a therapeutic anti-HBV drug is limited due to its toxicities.[20]

In order to gain a better insight into the structural requirements for potent and selective anti-HBV activity as a part of our ongoing antiviral research program, a number of 3′-fluoro (or chloro) pyrimidine nucleoside analogs were synthesized and evaluated to determine their effects against DHBV and HBV. Increasing number of HBV and HCV co-infections have also prompted us to search for agents that could be able to block the replication of both viruses, therefore, these compounds were investigated for their potential against HCV as well.

2. Chemistry

The 4-thio-3′-fluoro-2′,3′-dideoxyuridine (**1**), 5-trifluoromethyl-3′-fluoro-2′,3′-dideoxyuridine (**2**), 5-ethyl-3′-fluoro-2′,3′-dideoxyuridine (**3**), and 5-hydroxymethyl-3′-fluoro-2′,3′-dideoxyuridine (**4**) were prepared according to the published procedures.[21,22] The target 5-hydroxy pyrimidine nucleoside (**6**) was synthesized by the treatment of 3′-fluoro-2′,3′-dideoxyuridine (**5**)[23] with bromine–water followed by addition of pyridine at room temperature in 37% yield. (Scheme 1). Previously published methods of Balzarini et al.[24] were used to synthesize 5-iodo-3′-fluoro-2′,3′-dideoxyuridine (**7**) and 5-fluoro-3′-fluoro-2′,3′-dideoxyuridine (**8**). 3′,5-Difluoro-4-thio-2′,3′-dideoxyuridine (**12**) was prepared as illustrated in Scheme 2. Thus, reaction of **9** with trityl chloride and 4-(dimethylamino)pyridine in dry pyridine provided 5′-*O*-tritylated derivative **10** in 81% yield which upon thiation with P_2S_5 in pyridine gave the 4-thio nucleoside **11**. Compound **11** after detritylation with 80% aqueous AcOH at room temperature afforded **12** in 71% yield. 1-(2,3-Dideoxy-2,3-difluoro-β-D-ribofuranosyl)uracil (**15**) was synthesized in 37% yield by the reaction of 1-(3-fluoro-3-deoxy-β-D-arabinofuranosyl)uracil (**13**) with diethylaminosulfur trifluoride in benzene at −5 °C followed by subsequent detritylation of the obtained product **14** using 80% aqueous AcOH (Scheme 3). Pyrimidine nucleoside analogs, 1-(2,3-dideoxy-2,3-difluoro-β-D-ribofuranosyl)thymine (**16**) and 1-(3-chloro-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)thymine (**17**) were prepared according to the method reported by Huang et al.[25] and Verheyden et al.[26] The 1-(3-chloro-2,3-dideoxy-β-D-*threo*-pentofuranosyl)-5-fluorouracil (**18**) and 1-(3-chloro-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)-5-fluorouracil (**19**) were synthesized according to the procedures reported by us earlier.[27]

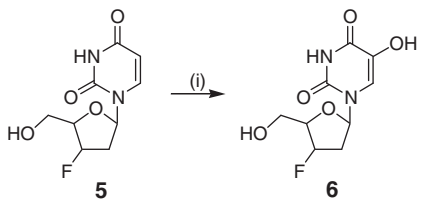
3. Results and discussion

The synthesized 3′-halo or 2′,3′-dihalo nucleosides (**1–4, 6–8, 12**, and **15–19**) were screened against duck hepatitis B virus (DHBV) in confluent cultures of primary duck hepatocytes obtained from chronically infected Pekin ducks.[28,29] The anti-HBV activity of the compounds **1**–**4**, **6**–**8**, **12**, and **15–19** 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.[30] 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}) are shown in Table 1. Lamivudine (3-TC) was used as a reference antiviral drug. As shown in Table 1, among the 3′-fluorinated compounds, 5-hydroxymethyl (**4**) and 5-iodo (**7**) exhibited significant anti-DHBV activity with EC_{50} values of 1 and 5 μg/mL, respectively. The 5-unsubstituted (**1**) and 5-trifluoromethyl (**2**) analogs showed weak inhibition and 5-ethyl (**3**), 5-hydroxy (**6**), and 5-fluoro (**8 and 12**) did not show any activity at 10 μg/mL. In these studies, 5-hydroxymethyl-3′-fluoro-2′,3′-dideoxyuridine (**4**) emerged as the most active inhibitor of DHBV replication that was 20-times less active than the reference drug 3-TC. The 2′,3′-difluoro nucleosides (**15 and 16**) were not found to possess anti-HBV activity. The inactivity of 2′,3′-difluoro-2′,3′-deoxythymidine (**16**) was surprising. Although 3′ fluoro-2′,3′-deoxythymidine (FddThd) is known to be an excellent anti-DHBV and anti-HBV agent, replacement of the 2′-hydrogen with a fluorine atom in FddThd (as in compound **16**) completely abrogated the anti-HBV activity despite the fact that the fluorine atom is similar to a hydrogen atom in terms of van der waal's radii. The 3′-chloro analog of 2′,3′-dideoxythymidine (**17**) was found to exhibit moderate anti-HBV activity (EC_{50} = 10 μg/mL) suggesting that substitution of 3′-fluoro by a chloro group is detrimental to anti-HBV activity. This observation was further supported by the fact that a 3′-chloro derivative of 5-fluoro-2′,3′-dideoxyurdine (**18**) was also devoid of anti-HBV activity. However, it is interesting to note that when the 3′-chloro substituent was inverted in the 'up' configuration (as in compound **19**), it leads to significant anti-DHBV activity (EC_{50} = 10 μg/mL). In our next studies, it would be interesting to investigate the anti-HBV activity of the 2′,3′-dideoxythymidine analog possessing a 3′-chloro substituent in *erythro* configuration.

Similar SARs were made when the compounds **1–4, 6**–**8**, **12**, and **15–9** were screened against human HBV in 2.2.15 cells,[31] except that the antiviral activity of compound **4** was reduced compared to that against DHBV. In this system, compounds **4**, **7, 17**, and **19** showed the best inhibition of HBV (Table 1).

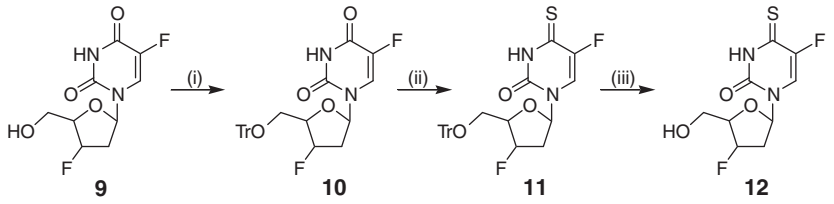
The anti-HCV activity of synthesized compounds was evaluated in the HCV RNA subgenomic replicon system in Huh-7 cells.[32] Pyrimidine nucleosides **1**–**4**, **6**–**8**, **12**, and **15–19** were initially screened at 20 μg/mL concentration (Table 2). In these studies, we observed that compounds **2**, **4**, **7**, **8**, and **19** exhibited appreciable inhibition of HCV replicon. The iodo derivative (**7**) exhibited 40% inhibition of HCV at 20 μg/mL, however, a concomitant decrease in cellular RNA levels was also observed (data not shown).

The exact mechanism of action of compounds inhibiting both HBV and HCV replication is not clear, however, it is possible that compounds **2**, **4**, **7**, and **19** exhibiting both anti-HBV and anti-HCV activities undergo phosphorylation by cellular kinases and their triphosphates can inhibit both HBV and HCV polymerases, and/or due to lack of 2′- and 3′-OH groups, they may also lead to viral DNA and RNA chain termination by acting as substrates of both viral polymerases. Although HBV and HCV belong to two different virus families and have DNA and RNA as their genomes, the replication of viral genome involves reverse transcription and DNA dependent DNA synthesis, and RNA dependent RNA synthesis, respectively. By the nature of general mechanism of action of modified nucleosides, it is possible to find analogs which can inhibit different enzymes found in these viruses and provide both anti-HBV and anti-HCV activities.

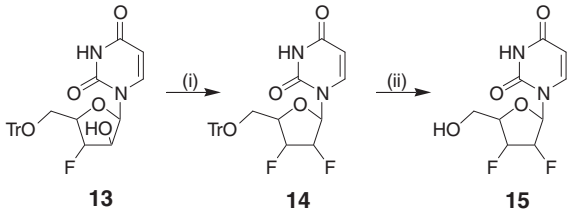


5 6

Scheme 1. Reagents and conditions: (i) Br_2–H_2O, H_2O, room temperature; pyridine, room temperature, 22 h, 37%.



Scheme 2. Reagents and conditions: (i) trityl chloride, 4-(dimethylamino)pyridine, dry pyridine, 80 °C, 8 h, 81% (for **10**); (ii) P_2S_5, pyridine–H_2O, reflux, 8 h, 27% (for **11**); (iii) 80% AcOH, room temperature, 72 h, 71% (for **12**).



Scheme 3. Reagents and conditions: (i) diethylaminosulfur trifluoride, anhydrous benzene, −5 °C to room temperature, 3 h, 39% (for **14**); (ii) 80% aq acetic acid, 90 °C, 30 min, 37% (for **15**).

In conclusion, from these studies, 5-hydroxymethyl-3′-fluoro-2′,3′-dideoxyuridine (**4**) and 1-(3-chloro-2,3-dideoxy-β-D-*erythro*-pentofuranosyl)-5-fluorouracil (**19**) emerged as interesting compounds that displayed significant anti-HBV activity and notable inhibition of hepatitis C replicon without causing cytotoxicity to host cells.

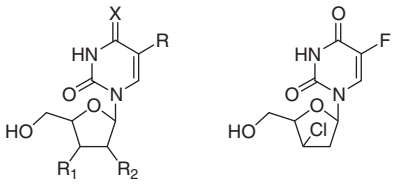
4. Experimental

Melting points were determined on Electrothermal melting point apparatus and are uncorrected. ^{1}H NMR spectra were determined for samples in Me_2SO-d_6 or $CDCl_3$ on a Bruker AM 300 spectrometer. Chemical shifts are given in ppm relative to TMS as an internal standard and signals are described as s (singlet), d (doublet), t (triplet), br s (broad signal), m (multiplet), dm (doublet of multiplet) and dd (doublet of doublets). The assignment of all exchangeable protons (OH, NH) was confirmed by the addition of the D_2O. Microanalysis results 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 (TLC) was performed with Machery-Nagel Alugram SiL G/UV silica gel slides (20 μM thickness).

4.1. 5-Hydroxy-3′-fluoro-2′,3′-dideoxyuridine (6)

A freshly prepared solution of bromine in water was added drop-wise to a solution of compound **5** (0.50 g, 2.17 mmol) in water (40 mL) at 25 °C with stirring until a yellow color persisted. TLC showed that the starting material was consumed. Excess bromine was evaporated through aeration. Pyridine (15 mL) was added slowly to the colorless reaction mixture, so that the temperature did not exceed 25 °C. The reaction mixture was stirred at room temperature for 22 h. Solvent was evaporated in vacuo followed by co-evaporation with ethanol. The residue thus obtained

Table 1
Antiviral activities of pyrimidine nucleosides against duck HBV and human HBV

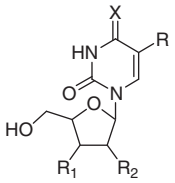


1-4, 6-8, 12, 15-18 **19**

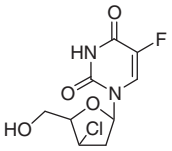
No.	R	R_1	R_2	X	DHBV primary duck hepatocytes % inhibition[a] (concd μg/mL)	EC_{50}[b] (μg/mL)	2.2.15 HBV % inhibition[a] (concd μg/mL)	EC_{50}[b] (μg/mL)
1	H	F	H	S	25 (10)		20 (10)	
2	CF_3	F	H	O	20 (10)		20 (10)	
3	C_2H_5	F	H	O	0		0	
4	CH_2OH	F	H	O	75 (10), 50 (1)	1	58 (10), 50 (5)	5–10
6	OH	F	H	O	0		0	
7	I	F	H	O	63 (10), 50 (5)	5	60 (10), 50 (5)	5
8	F	F	H	O	0		0	
12	F	F	H	S	0		0	
15	H	F	F	O	0		0	
16	CH_3	F	F	O	0		0	
17	CH_3	Cl	H	O	55 (10)	10	56 (10)	10
18	F	Cl	H	O	0		0	
19	—	—	—	—	50 (10)	10	50 (10)	10
3-TC[c]	—	—		—	96 (0.5–1.0)	0.05	88 (0.5–1)	0.2

[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] (−)-β-L-2′,3′-Dideoxy-3′-thiacytidine (Lamivudine, 3-TC).

Table 2
Anti-hepatitis C virus (HCV) activity of pyrimidine nucleoside analogs in the stably HCV RNA replicon expressing Huh7 cell line containing HCV 1a subtype



1-4, 6-8, 12, 15-18



19

Compd	R	R_1	R_2	X	Anti-HCV activity HCV 1a % inhibition[a] (μg/mL)	Viability (XTT assay) CC_{50}[b] (μg/mL)
1	H	F	H	S	0 (20)	>100
2	CF_3	F	H	O	25 (20)	>100
3	C_2H_5	F	H	O	0 (20)	>200
4	CH_2OH	F	H	O	30 (20)	>200
6	OH	F	H	O	0 (20)	>100
7	I	F	H	O	40 (20)	<100
8	F	F	H	O	28 (20)	>100
12	F	F	H	S	0 (20)	>100
15	H	F	F	O	0 (20)	>200
16	CH_3	F	F	O	0 (20)	>100
17	CH_3	Cl	H	O	0 (20)	>200
18	F	Cl	H	O	0 (20)	>200
19	—	—	—	—	30 (20)	>200
Ribavirin					50 (20)	ND[c]

[a] Anti-HCV activity was determined at concentrations 20 μg/mL.
[b] Concentration required to reduce Huh-7 cell viability by 50%.
[c] ND = not determined.

was purified on a silica gel column using EtOAc as eluent to give **6** (450 mg) as an impure solid, which was crystallized with MeOH/acetone to yield pure **6** (200 mg, 37%) as solid; mp 198–200 °C (dec); $[\alpha]_D$ −9.8 (*c* 0.40, DMSO); ^{1}H NMR (DMSO-d_6) δ 2.13–2.47 (m, 2H, H-2′), 3.52–3.66 (m, 2H, H-5′), 4.14 (dm, $J_{H\text{-}4',3'\text{-}F}$ = 25.02 Hz, 1H, H-4′), 5.21 (t, *J* = 4.58 Hz, 1H, 5′-OH), 5.29 (dd, $J_{H\text{-}3',3'\text{-}F}$ = 53.10 Hz, 4.27 Hz, 1H, H-3′), 6.19–6.25 (m, 1H, H-1′), 7.37 (s, 1H, H-6), 8.75 (s, 1H, 5-OH), 11.52 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 36.66 (d, C-2′, *J* = 20.12 Hz), 60.93 (d, C-5′, *J* = 11.16 Hz), 83.73 (C-1′), 84.64 (d, C-4′, *J* = 22.63 Hz), 94.95 (d, C-3′, *J* = 173.60 Hz), 119.03 (C-6), 132.68 (C-5), 149.07 (C-2), 160.41 (C-4). Anal. Calcd for $C_9H_{11}FN_2O_5 + 0.9H_2O$: C, 41.20; H, 4.92; N, 10.68. Found: C, 41.48; H, 5.01; N, 10.33.

4.2. 3′,5-Difluoro-5′-*O*-trityl-2′,3′-dideoxyuridine (10)

A mixture of 3′,5-difluoro-2′,3′-dideoxyuridine (**9**, 2.0 g, 8.06 mmol), trityl chloride (3.37 g, 12.09 mmol), and 4-(dimethylamino)pyridine (0.1 g, 0.82 mmol) in dry pyridine (40 mL) was heated at 80 °C for 8 h. The solvent was removed in vacuo and the crude product thus obtained was purified on silica gel column using MeOH/$CHCl_3$ (4:96, v/v) as eluent to give **10** (3.20 g, 81%) as a sirup; ^{1}H NMR ($CDCl_3$) δ 2.18–2.41 (m, 1H, H-2′), 2.73–2.87 (m, 1H, H-2″), 3.40–3.57 (m, 2H, H-5′), 4.38 (dm, $J_{H\text{-}4',3'\text{-}F}$ = 27.16 Hz, 1H, H-4′), 5.32 (dm, $J_{H\text{-}3',3'\text{-}F}$ = 53.71 Hz, 1H, H-3′), 6.34–6.41 (m, 1H, H-1′), 7.28–7.50 (m, 15H, 5′-*O*-trityl), 7.81 (d, *J* = 6.10 Hz, 1H, H-6), 8.96 (br s, 1H, NH); ES-MS (+ve mode) = 513.1 $(M+Na)^+$; ES-MS (−ve mode) = 489.2 $(M-1)^+$.

4.3. 3′,5-Difluoro-4-thio-5′-*O*-trityl-2′,3′-dideoxyuridine (11)

A mixture of **10** (1.80 g, 3.67 mmol), phosphorus pentasulfide (2.93 g, 6.59 mmol), pyridine (100 mL), and water (0.1 mL) was refluxed for 14 h and then cooled to room temperature. The solvent was removed in vacuo and ice-water mixture was added to the residue. The product was extracted with $CHCl_3$ (3 × 50 mL). Combined organic layer was dried over anhydrous Na_2SO_4 and concentrated to give the crude product which was purified on silica gel column using $CHCl_3$ as eluent to give **11** (0.50 g, 27%) as a sirup; ^{1}H NMR (DMSO-d_6) δ 2.38–2.68 (m, 2H, H-2′), 3.21–3.42 (m, 2H, H-5′), 4.27 (dm, $J_{H\text{-}4',3'\text{-}F}$ = 27.17 Hz, 1H, H-4′), 5.33 (dm, $J_{H\text{-}3',3'\text{-}F}$ = 52.49 Hz, 1H, H-3′), 6.06–6.11 (m, 1H, H-1′), 7.26–7.47 (m, 15H, 5′-*O*-trityl), 7.89 (d, *J* = 4.58 Hz, 1H, H-6), 13.16 (s, 1H, NH); ES-MS (+ve mode) = 529.1 $(M+Na)^+$; ES-MS (−ve mode) = 505.1 $(M-1)^+$.

4.4. 3′,5-Difluoro-4-thio-2′,3′-dideoxyuridine (12)

The compound **11** (0.50 g, 0.99 mmol) in 80% acetic acid (20 mL) was stirred at room temperature for 72 h. Solvent was removed in vacuo and the obtained crude product was purified on silica gel column using EtOAc/hexane (40:60, v/v) as eluent to give **12** (0.185 g, 71%) as sirup that could not be crystallized out using ethanol; $[\alpha]_D$ +65.04 (*c* 0.36, MeOH); ^{1}H NMR (DMSO-d_6) δ 2.20–2.61 (m, 2H, H-2′), 3.58–3.70 (m, 2H, H-5′), 4.23 (dm, $J_{H\text{-}4',3'\text{-}F}$ = 27.16 Hz, 1H, H-4′), 5.29 (dm, $J_{H\text{-}3',3'\text{-}F}$ = 53.40 Hz, 1H, H-3′), 5.33 (t, *J* = 4.89 Hz, 1H, 5′-OH), 6.09–6.16 (m, 1H, H-1′), 8.18 (d, *J* = 4.58 Hz, 1H, H-6), 13.13 (s, 1H, NH); ^{13}C NMR (CD_3OD) δ 39.65 (d, C-2′, *J* = 20.52 Hz), 62.58 (d, C-5′, *J* = 11.06 Hz), 87.24 (C-1′), 87.45 (d, C-4′, *J* = 23.94 Hz), 95.99 (d, C-3′, *J* = 175.61 Hz), 121.69 (d, C-6, *J* = 41.64 Hz), 148.68 (C-2), 148.86 (d, C-5, *J* = 222.18 Hz), 182.77 (d, C-4, *J* = 30.88 Hz). Anal. Calcd for $C_9H_{10}F_2N_2O_3S + 0.3C_2H_5OH$: C, 41.47; H, 4.28; N, 10.07; S, 11.53. Found: C, 41.61; H, 4.00; N, 10.38; S, 11.13.

4.5. 1-(2,3-Dideoxy-2,3-difluoro-5-*O*-trityl-β-D-ribofuranosyl)uracil (14)

Diethylaminosulfur trifluoride (DAST) (0.95 g, 5.9 mmol) was added to a solution of **13** (0.41 g, 0.82 mmol) in benzene (10 mL) at −5 °C. The reaction mixture was stirred at room temperature for 24 h and poured into 5% aqueous $NaHCO_3$ (w/v, 10 mL) dropwise.

The aqueous portion was extracted with EtOAc (3 × 50 mL). The organic layer was dried over Na_2SO_4 and concentrated in vacuo. The residue was purified on a silica gel column using MeOH/$CHCl_3$ (2:98, v/v) as eluent to yield **14** (0.16 g, 39%) as a sirup; ^{1}H NMR (DMSO-d_6) δ 3.32 (m, 2H, H-5′), 4.23–4.39 (m, 1H, H-4′), 5.31–5.50 (m, 1H, H-3′), 5.52 (dd, J = 7.93 Hz and 1.88 Hz, 1H, H-5), 5.54–5.67 (m, 1H, H-2′), 5.96 (d, J = 18 Hz, 1H, H-1,), 7.16–7.47 (m, 15H, trityl), 7.72 (d, J = 8.54 Hz, 1H, H-6), 11.53 (s, 1H, NH); ES-MS (+ve mode) = 513.1 $(M+Na)^+$; ES-MS (−ve mode) = 489.2 $(M-1)^+$.

4.6. 1-(2,3-Dideoxy-2,3-difluoro-β-D-ribofuranosyl)uracil (15)

A solution of **14** (0.15 g, 0.31 mmol) in 80% aqueous acetic acid (10 mL) was heated at 90 °C for 30 min. The solvent was removed in vacuo. The residue was purified on a silica gel column using MeOH/$CHCl_3$ (5:95, v/v) as eluent to yield **15** (0.028 g, 37%) as a sirup; $[\alpha]_D$ −20.87 (*c* 0.23, MeOH); ^{1}H NMR (DMSO-d_6) δ 3.62 (m, 1H, H-5′), 4.27 (dm, J = 22.58 Hz, 1H, H-4′), 5.27 (dm, J = 53.10 Hz, 1H, H-3′), 5.38 (m, 1H, 5′-OH), 5.39 (dm, J = 51.26 Hz, 1H, H-2′), 5.71 (d, 1H, H-5, J = 7.93 Hz), 6.06 (dd, J = 15.2 Hz and 4.88 Hz, 1H, H-1′), 7.85 (d, J = 7.93 Hz, 1H, H-6), 11.50 (s, 1H, NH); ^{13}C NMR CD3OD: δ 61.61 (C-5′), 84.12 (C-4′, J = 22.7 Hz), 88.25 (C-1′, J = 32.6 Hz), 90.17 (C-3′, J = 163.90 Hz and 14.4 Hz), 92.07 (C-2′, J = 169.60 Hz and 14.4 Hz), 103.40 (C-5), 142.51 (C-6), 152.19 (C-2), 165.90 (C-4). Anal. Calcd for $C_9H_{10}F_2N_2O_4$: C, 43.55; H, 4.06. Found: C, 43.95; H, 4.48.

4.7. 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.[28,29] Pekin duck eggs were obtained from a duck colony maintained at the University of Alberta farm and were stored in a 37 °C egg incubator until hatching occurred. Livers from congenitally DHBV infected ducks were used. Persistently infected ducks were identified by detection of DHBV DNA in sera by dot hybridization.[28,29] Primary cultures of duck hepatocytes were prepared from 9 to 14 day old DHBV-infected ducklings using a modified method.[28,29] Cells were cultured in 6-well plates in 3 mL L-15 medium containing 5% fetal bovine serum, penicillin G sodium (10 IU/mL), and streptomycin sulfate (10 μg/mL). The test compounds were added in triplicate to the hepatocyte cultures on day 2, and were maintained in culture with media changed every second day until day 16. Cells were harvested at day 17. Initially, the test compounds were screened at a 10 μg/mL final concentration. Inhibition in DHBV replication at 10 μg/mL was calculated as the average of triplicate wells. Standard deviations were within 10% of the average values. After initial testing, the compounds were serially diluted to determine more precise anti-DHBV EC_{50} values. The hepatocytes were lysed with 1.0 mL lysis buffer containing 10 mM Tris–HCl and 1% SDS. The lysate was digested with 0.2 mg proteinase K and extracted with an equal volume of phenol saturated with Tris–HCl EDTA and 0.1% 8-hydroxyquinoline, followed by extraction with chloroform. Concentrated NaCl (5 M) was added to the aqueous phase to yield a final concentration of 0.5 M NaCl, and the DNA was precipitated with two volumes of 95% ethanol. The DNA pellet was washed with 70% ethanol and dried. The dried DNA was dissolved in 50 μL of a solution containing Tris–HCl EDTA. DNA samples were applied to a nylon filter (Hybond-N, Amersham) using a Bio-Dot (Bio-Rad Laboratories) microfiltration apparatus. DNA on the filter was denatured with NaOH/NaCl at room temperature for 30 min and neutralized in Tris–HCl/NaCl. The filters were exposed to ultraviolet irradiation for 3 min. DNA hybridization was initiated by adding a recently prepared DHBV (^{32}P) DNA probe at 10^6 CPM/mL and incubating overnight. Filters were washed twice in 1 × SSC (20 × SSC = 3 M NaCl plus 0.3 M sodium citrate, pH 7.0)-0.1% SDS at 65 °C for 30 min and 0.1 SSC, 0.15 DS at room temperature for 30 min. After an autoradiographic image was obtained, the filters were exposed in a phosphoimaging screen for 1–2 h, samples were quantitated by a Fujix BAS1000, and the percentage density of phosphoimaging units were calculated.20 Lamivudine was used as the reference compound. Tests were repeated two to three 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 two to three experiments was within 10% standard deviation, average values are shown, otherwise a range of EC_{50} values are shown. Percent inhibition was calculated by using the formula:

$$\%\text{Inhibition} = \frac{\text{Untreated positive control} - \text{treated test sample}}{\text{Untreated positive control}} \times 100$$

4.8. 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.[30,31] These cells were derived from HepG2 cells that were transfected with a plasmid vector containing G418-resistant sequences and two head to tail dimers of the HBV genome. The cell culture medium consisted of MEM (Sigma), supplemented with 10% heat inactivated FBS (Gibco), geneticin/G148 sulfate (380 μg/mL), 5–7 mL of 7.5% sodium bicarbonate, and L-glutamine (2 mM). Initially, the test compounds were screened at a 10 μg/mL final concentration. Inhibition of HBV replication at 10 μg/mL was calculated as the average of triplicate wells. Standard deviations were within 10% of the average values. After initial testing, the compounds were serially diluted to determine more precise anti-HBV EC_{50} values. The 2.2.15 cells were grown in MEM media in a humidified 37 °C incubator with a 5% CO_2 atmosphere and seeded into 6-well plates overnight to obtain a confluent monolayer. The compounds were tested in triplicate for each concentration. The drug solutions were added at a volume of 3 mL per well, replacing the prior media. The media containing test compounds was replaced every second day, for four to five treatments in total. On the day subsequent to the last treatments, the cells were harvested for the analysis of intracellular HBV DNA. The method for isolated intracellular DNA and dot-blot hybridization were essentially similar to that for DHBV, except human HBV specific radioactive probe was used. Percent inhibition was calculated by using the formula: (Untreated positive control − treated test sample) × 100/Untreated positive control. Tests were repeated two to three 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.

4.9. In vitro antiviral assay for HCV

Anti-HCV activity was determined in HCV 1a subgenomic replicon cells as described previously.[32] Briefly, 1×10^5 replicon cells per well were plated in 24-well plates. On the next day, replicon cells were incubated at 37 °C with test compounds at 20 μg/mL. The cells were treated with fresh media containing test compounds every second day for a total of three to four treatments. Total cellular RNA was extracted using an RNAeasy-96 kit (Qiagen, Valencia, CA), cDNA was synthesized using iScript cDNA synthesis kit (BioRAD, CA) and the

copy number of HCV RNA was determined using a quantitative RT-PCR (QRT-PCR) assay.[33] β-Actin was used to normalize the HCV RNA copy numbers. The primers used for PCR assays were HCV UTR F: 5′-CTG TCT TCA CGC AGA AAG CG-3′, HCV UTR R: 5′-CAC TCG CAA GCA CCC TAT CA-3′, β-actin F: 5′-CGA TGC AGA AGG AGA TCA CTG-3′, β-actin R: 5′-CGA TCC ACA CGG AGT ACT TG-3′. The % inhibition shown is average of triplicates and the standard deviations were within 10%. Ribavirin at 20 μg/mL was used in the same conditions as a positive control.

4.10. Cell cytotoxicity assay

Human hepatoma cell line (Huh-7) was used to determine the effect of test compounds on human cell cytotoxicity using the XTT assay. Cell viability was measured using the cell proliferation kit II (XTT; Roche), as per manufacturer's instructions. Briefly, a 96 well plate was seeded with Huh-7 cells at a density of 1×10^5 cells per well. Cells were allowed to attach for 6–8 h when the medium was replaced with medium containing compounds at concentrations of 200, 100, 50, 10, and 1 μg/mL. DMSO was also included as control. Plates were incubated for 2 days at 37 °C. The color reaction involved adding 50 μL XTT reagents per well and incubating for 4 h at 37 °C. Plates were read on an ELISA plate reader (Abs 450–500 nm).

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