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Research paper

Design, synthesis, and biological evaluation of new N^4-Substituted 2′-deoxy-2′-fluoro-4′-azido cytidine derivatives as potent anti-HBV agents

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ABSTRACT

A series of new 2′-deoxy-2′-β-fluoro-4′-azido-β-D-arabinofuranosyl cytidine derivatives bearing heteroatom-containing N^4-substituents were designed and synthesized. Antiviral screening in HepG2.2.15 cells identified three analogs (**1a**, **1d** & **1g**) with good anti-HBV activity and low cytotoxicty. Of them, compound **1g** exhibited significant inhibitory activity on both HBV antigens secretion ($EC_{50,\ HBsAg}$ = 9 nM, $EC_{50,\ HBeAg}$ = 0.25 μM) and viral DNA replication (intracellular, EC_{50} = 0.099 μM; extracellular, EC_{50} < 0.01 μM).

1. Introduction

Globally, hepatitis B remains a major infectious disease, which affects more than 240 million individuals chronically worldwide and causes as many as 780,000 deaths a year [1]. Currently, there are only seven antiviral drugs in clinical use against hepatitis B virus (HBV) infection: two interferon-α (IFN-α) products as immune system modulators and five nucleoside/nucleotide analogs as viral polymerase inhibitors. However, due to limited efficacy, side effects and/or emergence of resistance, the current therapeutic options still cannot meet the clinic need for the treatment of chronic hepatitis B. Thus, it is urgent to develop new and effective anti-HBV drugs whether for monotherapy or combination therapy. Although a number of non-nucleoside compounds have been discovered with good anti-HBV activity [2–8], so far, none of them has got the FDA approval for clinical use. Compared with interferon-α, nucleosides/nucleotides are orally available and effective in almost all the patients, while causing fewer side effects, which is still one of the most promising areas for developing new potential anti-HBV therapeutics.

Our research group has been working on the design and discovery of novel nucleosides against human immunodeficiency virus (HIV) [9–12], HBV [13–16], and hepatitis C virus (HCV) [17]. Previously, we reported the discovery of 2′-deoxy-2′-β-fluoro-4′-azido-β-D-arabinofuranosyl cytidine (FNC, **6**) with good anti-HBV activity both in vitro [16] and in vivo [15]. In a recent research work, it has been demonstrated that N^4-alkylation (*e.g.* compound **7**) of nucleoside **6** could significantly reduce the in vivo toxicity of the compound [14]. However, these N^4-alkylated analogs are less

Abbreviations used: HBV, hepatitis B virus; IFN, interferon; FDA, food and drug administration; HIV, human immunodeficiency virus; HCV, hepatitis C virus; TPSCl, 2,4,6-triisopropylbenzenesulfonyl chloride; DIPEA, disopropylethylamine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CC_{50}, 50% cytotoxic concentration; EC_{50}, 50% effective concentration; 3 TC, lamivudine; ETV, entecavir; PCR, polymerase chain reaction; FQ-PCR, fluorescence quantitative PCR; HBsAg, HBV surface antigen; HBeAg, HBV e antigen; DNA, deoxyribonucleic acid; ELISA, enzyme-linked immunosorbent assay; TLC, thin layer chromatography; MEM, minimum essential medium.

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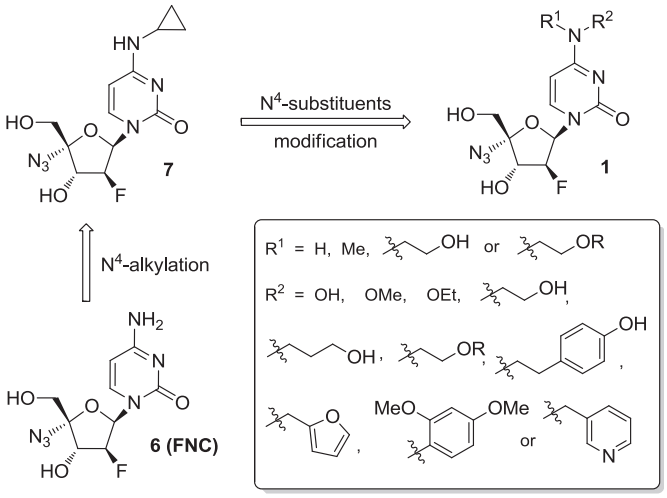


Scheme 1. Structure Exploration of the N^4-Alkylated FNC Derivatives.

potent than the parent compound. Herein, we carried out further structural exploration of the N^4-substituents by incorporating oxygen atoms as hydroxyl groups or ether bonds (Scheme 1), aiming at further increasing the antiviral potency. The biological activity of all the new cytidine derivatives was evaluated in HepG2.2.15 cell lines.

2. Results and discussion

2.1. Chemistry

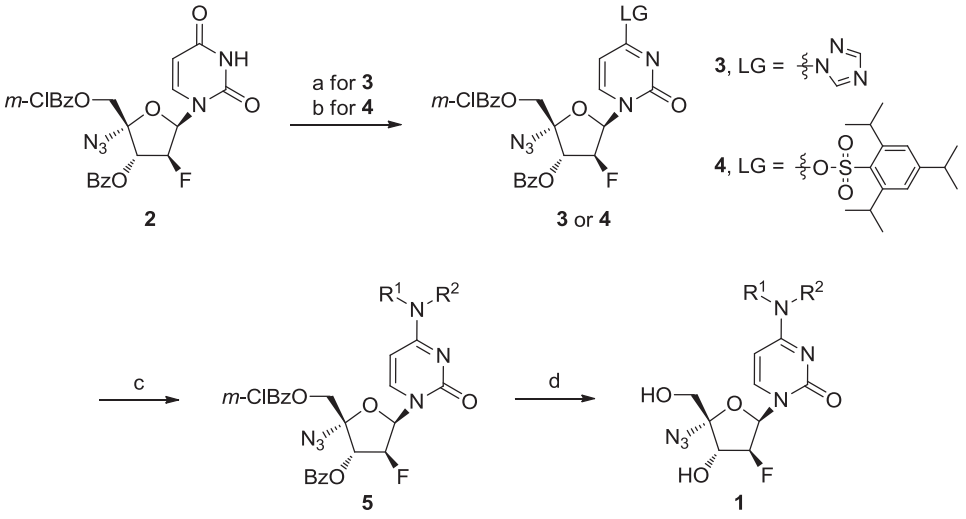
The target nucleosides **1** were synthesized as outlined in Scheme 2. The key starting material, bisbenzoyl uridine **2** was prepared from 1,3,5-*O*-tribenzoyl-2-deoxy-2-fluoro-D-arabinofuranoside using a previously reported synthetic route [10]. The 4-carbonyl group in compound **2** was selectively activated by reaction with 1,2,4-triazole [14] or 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl) [18] to form intermediates **3** or **4**. Treatment of compound **3** (or **4**) with substituted amines in the presence of disopropylethylamine (DIPEA) followed by deprotection with ammonia in methanol afforded compound **1a**–**l** in 39–75% yield. All these new synthesized nucleoside analogs were fully characterized by ^{1}H NMR, ^{13}C NMR and HRMS. Analytical HPLC indicated that all the tested compounds possess a purity of at least 95%.

2.2. Cytotoxicity

In the HepG2.2.15 cell line, the cytotoxicity of these new cytidine derivatives (**1a**–**l**) was measured by an MTT assay. As shown in Table 1, most of these nucleoside analogs (**1a**–**c** and **1f**–**j**) exhibited further reduced toxicity compared to the parent compound **7**. Among them, two compounds (**1a** and **1g**) are less toxic than the positive control drug lamivudine (3 TC, CC_{50} = 859 μM). In particular, no cytotoxicity was observed for the bis(2-hydroxyethyl) amino analog **1g** up to the highest concentration tested (1250 μM).

2.3. Anti-HBV activity screening

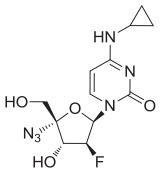
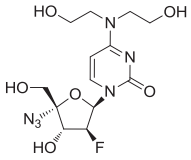
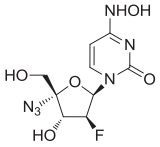
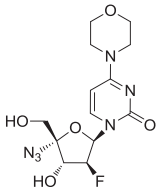
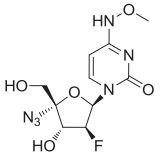
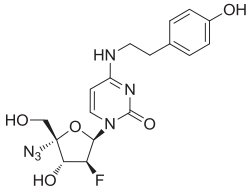
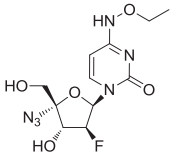
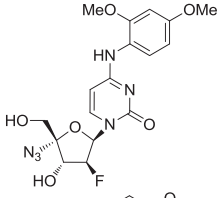
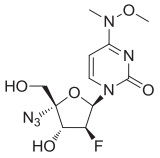
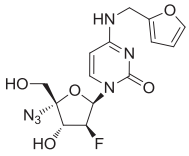
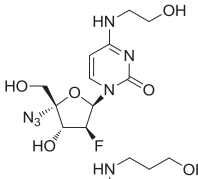
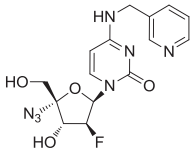
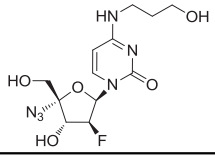
The anti-HBV activity of these nucleosides (**1a**–**l**) was firstly screened in the HepG2.2.15 cell line using entecavir (ETV) as a positive control. After the treatment of the cells with the test compounds at the concentration of 1 μM for 9 days, their inhibitory activity against HBV DNA replication was measured by an FQ-PCR assay (Table 2). Compared to the parent compound **7**, direct N^4-hydroxylation greatly increased the anti-HBV activity of the compound (**1a**). O-Alkylation of **1a** did not improve the biological activity, with the ethoxy cytidine **1c** being more potent than its methoxy analog **1b**. Further *N*-methylation of **1b** significantly decreased the biological activity of the compound (**1d**). Analogs bearing N^4-hydroxyalkyl groups (**1e**–**f**) maintained the good antiviral activity. Incorporation of one more hydroxyethyl group to the N^4-position of **1e** led to compound **1g** with significant potency against HBV DNA replication (96.9%). However, the morpholine analog (**1h**) completely lost the anti-HBV activity. Introduction of aromatic rings (e.g. benzene, furan and pyridine rings) to the N^4-



(a) 1,2,4-triazole, Py., $POCl_3$, CH_2Cl_2, reflux; (b) 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl), DIPEA, DMAP CH_2Cl_2, rt; (c) R^1R^2NH, DIPEA, DMAP (when **4** was used), CH_2Cl_2, rt; (d) sat. NH_3 in methanol, rt.

Scheme 2. Synthesis of Compounds **1** from Bisbenzoyl Protected Uridine **2.**

Table 1
Cytotoxicity of compounds **1**.[a]

Compound	Structure	CC_{50} (μM)	Compound	Structure	CC_{50} (μM)
7		391	**1g**		>1250
1a		951	**1h**		700
1b		418	**1i**		834
1c		572	**1j**		722
1d		>250	**1k**		<2
1e		<2	**1l**		>10
1f		668	3 TC	–	859

[a] CC_{50}: 50% cytotoxic concentration, measured by an MTT assay in the HepG2.2.15 cell line.

side-chain resulted in derivatives (**1i**–**l**) with poor antiviral activity. Overall, three compounds (**1a**, **1d** & **1g**) exhibited potent inhibitory activity against HBV DNA replication (97.0%, 93.9% and 96.9%), which are equally good or slightly better than that of compound **6** and the potent anti-HBV drug entecavir (ETV, 95.7%) at the same concentration tested.

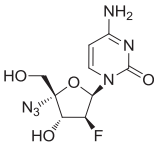
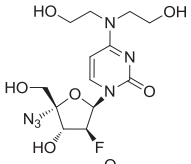
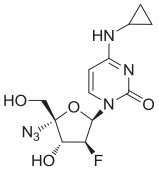
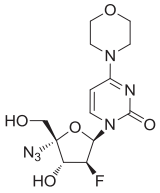
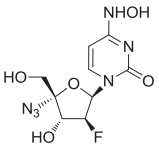
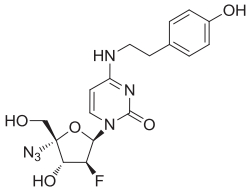
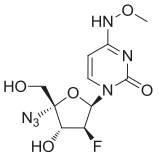
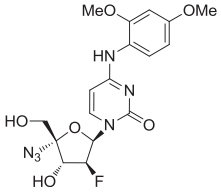
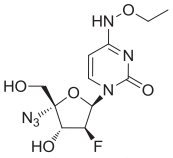
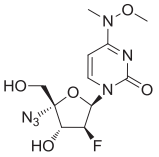
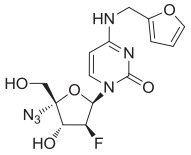
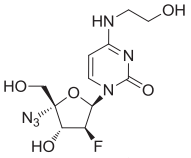
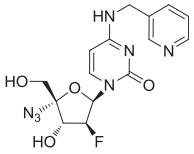
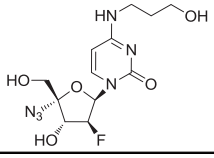
2.4. Inhibitory effect on HBsAg and HBeAg secretion

According to the SAR results above, the inhibitory effect of selected analogs (**1a**, **1d** & **1g**) on the secretion of HBsAg and HBeAg was further evaluated in the HepG2.2.15 cell line. The supernatant was collected after the cells were treated with these compounds at various concentrations (1, 0.1 and 0.01 μM), and the levels of HBsAg and HBeAg in the supernatant were detected by ELISA assays. Treatment of HepG2.2.15 cells with the test compounds resulted in significant reduction on both HBsAg and HBeAg secretion in a dose-dependent manner. As illustrated in Table 3, all the three nucleosides displayed nanomolar level inhibitory activity on HBsAg secretion on Day 9, with the bis(2-hydroxyethyl)amino analog **1g** being the most potent one (EC_{50} = 9 nM). In addition, compound **1g** also exhibited an EC_{50} of 0.25 μM on the inhibition of HBeAg secretion. On Day 9, the positive control drug lamivudine (3 TC) at the concentration of 25 μM only showed 43.4% and 46.0% inhibition of HBsAg and HBeAg, respectively.

2.5. Inhibitory effect of compound 1g on HBV DNA replication

Consistent with the inhibitory activity on HBV antigens secretion, compound **1g** also reduced both extracellular and intracellular HBV DNA levels in HepG2.2.15 cells in a dose-dependent manner. On Day 9, the average inhibition of viral DNA levels at the dosages

Table 2
Inhibitory effect of compounds **1** on HBV DNA replication.[a]

Compound	Structure	Inhibition rate (1 μM)	Compound	Structure	Inhibition rate (1 μM)
6		98.6%	**1g**		96.9%
7		86.6%	**1h**		<10%
1a		97.0%	**1i**		<10%
1b		81.2%	**1j**		<10%
1c		93.9%			
1d		34.0%	**1k**		53.6%
1e		82.1%	**1l**		24.3%
1f		77.6%	ETV	—	95.7%

[a] HepG2.2.15 cells were treated with the test compounds at the concentration of 1.0 μM and the inhibition of HBV DNA replication was measured by an FQ-PCR assay.

of 1, 0.1 and 0.01 μM was 61.9%, 51.8% and 36.4% intracellularly (Fig. 1A, EC_{50} = 0.099 μM) and 76.1%, 62.2% and 53.6% extracellularly (Fig. 1B, EC_{50} < 0.01 μM), respectively.

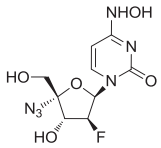
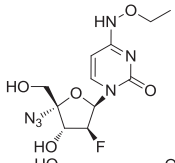
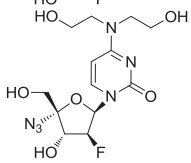
2.6. In silico prediction of physicochemical properties

Furthermore, some physicochemical properties of **1g**, its parent compounds (**6**–**7**), and 3 TC, ETV (as control) were predicted using free online software (http://www.molinspiration.com/) for their compliance with the Lipinski's rule of five. As shown in Table 4, **1g**, **6**–**7**, and reference compounds conformed well to the Lipinski's rule of five. Overall, compound **1g** exhibited potent in vitro anti-HBV activity, low toxicity and reasonable physicochemical properties, and thus could be considered as a promising candidate for further development.

3. Conclusions

Building upon the structures of cytidine nucleosides **6** and **7**, we have designed and synthesized a series of new analogs bearing heteroatom-containing N^4-substituents. Antiviral screening on HepG2.2.15 cell line identified three compounds (**1a**, **1d** & **1g**) with potent inhibitory activity against HBV DNA replication. Treatment of the cells with compound **1g** for 9 days resulted in significant

Table 3
Inhibitory Activity of selected compounds (**1a**, **1c**, and **1g**) on HBsAg and HBeAg secretion.[a]

Compound	Structure	EC_{50} (μM, HBsAg)	EC_{50} (μM, HBeAg)
1a		0.69	>1
1c		0.12	>1
1g		0.009	0.25

[a] EC_{50}: 50% effective concentration, measured by HBsAg and HBeAg ELISA assays, respectively, in the HepG2.2.15 cell line.

inhibition on the secretion of HBV antigens ($EC_{50,\ HBsAg}$ = 9 nM, $EC_{50,\ HBeAg}$ = 0.25 μM), and reduction of both intracellular (EC_{50} = 0.099 μM) and extracellular (EC_{50} < 0.01 μM) HBV DVA levels. In addition, no cytotoxicity was observed for this compound at the highest concentration tested (1250 μM) on the same cell line. These encouraging results justify its further development as a potential anti-HBV agent.

4. Experimental section

4.1. Chemistry

^{1}H NMR spectra were recorded on a 400 MHz (^{13}C NMR spectra were recorded on a 100 MHz) spectrometer. Chemical shift values are given in ppm and referred to the internal standard of TMS (tetramethylsilane). The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; quint, quintet; m, multiplet and dd, doublet of doublets. The coupling constants (*J*) are reported in Hertz (Hz). Flash column chromatography was performed over silica gel 200–300 mesh, and the eluent was a mixture of MeOH/CH_2Cl_2 or EtOAc/petroleum ether. High-resolution mass spectra (HRMS-ESI) were obtained on a Q-TOF Mass Spectrometer. All the tested compounds possess a purity of at least 95%. Analytical HPLC was run on the Agilent 1260 HPLC instrument, equipped with Agilent XDB-C18 column and UV detection at 270 nm. Eluent system was: 40% MeOH in H_2O; flow rate = 0.8 mL/min.

4.1.1. General Procedure A for the synthesis of nucleoside ***1*** *via intermediate* ***3***

The crude intermediate **3** was prepared as a pale yellow solid in nearly quantitative yield according to our previously reported procedure [14], which was used directly in the next step without further purification. Compound **3** (116 mg, 0.2 mmol) was dissolved in CH_2Cl_2 (2 mL), and then treated with the corresponding amine

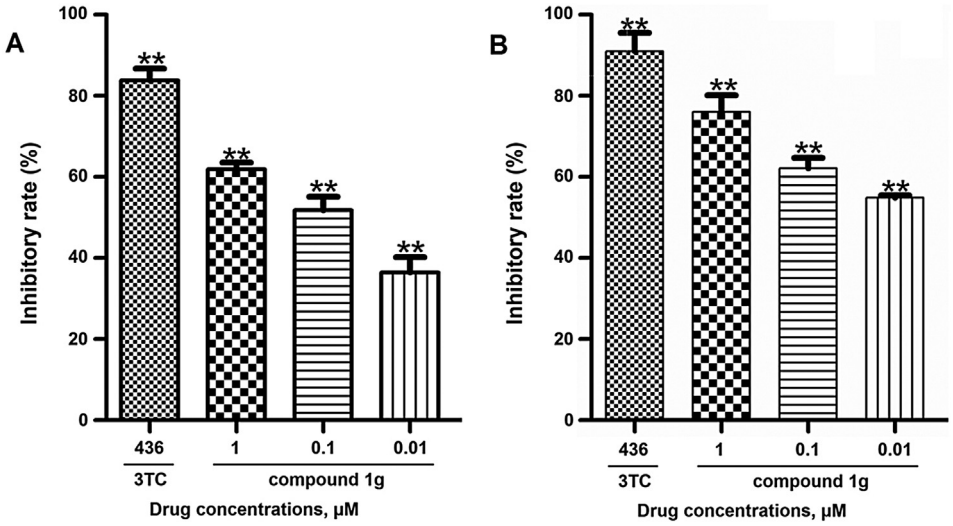


Fig. 1. Inhibitory effect of compound **1g** on HBV DNA level. HepG2.2.15 cells were cultured in the presence of compound **1g** at various concentrations (1, 0.1 and 0.01 μM) or 3 TC at 436 μM for 9 days, and then intracellular (A) and extracellar (B) HBV DNA levels were quantified by real-time FQ-PCR. The experiments were performed three times, **P < 0.01 compared to control.

Table 4
Prediction of physicochemical properties[a] of **1g**, **6**–**7**, 3 TC and ETV.

Compound	nViol	natoms	miLogP	MW/Da	nON	nOHNH	Nrotb	TPSA/Å^2	MV
Accepted range			<5	<500	<10	<5	≤10	<140	
1g	1	26	−1.84	374.33	12	4	8	178.04	308.05
6	1	20	−1.20	286.22	10	4	3	160.37	223.32
7	0	23	−0.46	326.29	10	3	5	146.37	264.02
3 TC	0	15	−1.09	229.26	6	3	2	90.38	187.07
ETV	0	20	−1.06	277.28	8	5	2	130.06	237.36

[a] nViol: no. of violations; natoms: no. of atoms; miLogP: molinspiration predicted LogP; MW: molecular weight; nON: no. of hydrogen bond acceptors; nOHNH: no. of hydrogen bond donors; nrotb: no. of rotatable bonds; TPSA: topological polar surface area; MV: molar volume.

(0.5 mmol) and *N*,*N*-diisopropylethylamine (DIPEA, 87 μL, 0.5 mmol; another 0.5 mmol of DIPEA was added, if the HCl salt of the amine was used). After stirring at room temperature for 4–12 h (TLC indicated that the conversion was complete), the reaction was quenched with 5% citric acid (10 mL) and stirred for 10 min. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (10 mL × 3). The combined organic layer was washed with brine (20 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by silica gel column chromatography to give the corresponding intermediate **5**, which was stirred in sat. NH_3 solution in MeOH (5 mL) until TLC indicated that the debenzoylation was complete. The solvent was evaporated, and the resulting residue was purified through silica gel column chromatography to afford products **1a**, **1d**–**f** and **1h**–**l** in 39–70% yield overall three steps.

*4.1.2. General Procedure B for the synthesis of nucleoside **1** via intermediate **4***

4-Dimethylaminopyridine (DMAP, 122 mg, 1 mmol) and DIPEA (6.97 mL, 40 mmol) were added sequentially to a suspension of compound **2** (2.65 g, 5 mmol) in anhydrous CH_2Cl_2 (30 mL). After compound **2** was completely dissolved, the resulting mixture was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (TPSCl, 3.03 g, 10 mmol). After stirring at room temperature for 24 h, the reaction mixture was concentrated and purified through silica gel column chromatography to give intermediate **4** as a white solid (3.90 g, 86%). Compound **4** (239 mg, 0.3 mmol) was dissolved in CH_2Cl_2 (3 mL), and then treated with the corresponding amine (0.9 mmol), DIPEA (157 μL, 0.9 mmol; another 0.9 mmol of DIPEA was added, if the HCl salt of the amine was used) and DMAP (7 mg, 0.06 mmol). After stirring at room temperature for 4–12 h (TLC indicated that the conversion was complete), the reaction was quenched with 5% citric acid (10 mL) and stirred for 10 min. The organic layer was separated and the aqueous layer was extracted with CH_2Cl_2 (10 mL × 3). The combined organic layer was washed with brine (20 mL), dried over anhydrous sodium sulfate, and concentrated. The resulting residue was purified by silica gel column chromatography to give the corresponding intermediate **5**, which was stirred in sat. NH_3 solution in MeOH (5 mL) until TLC indicated that the debenzoylation was complete. The solvent was evaporated, and the resulting residue was purified through silica gel column chromatography to afford products **1b**, **1c** and **1g** in 68–75% yield overall three steps.

*4.1.2.1. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-(hydroxyamino)pyrimidin-2(1H)-one (**1a**).* The product was obtained according to General Procedure A, as a white solid (53%); ^{1}H NMR (400 MHz, CD_3OD) δ 6.95 (dd, *J* = 8.4, 1.6 Hz, 1H), 6.39 (dd, *J* = 11.2, 5.6 Hz, 1H), 5.57 (d, *J* = 8.4 Hz, 1H), 5.13 (dt, *J* = 54.0, 5.6 Hz, 1H), 4.47 (dd, *J* = 22.8, 5.2 Hz, 1H), 3.82–3.80 (m, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 149.7, 144.4, 130.9 (d, $J_{C\text{-}F}$ = 2.7 Hz), 97.5, 96.3 (d, $J_{C\text{-}F}$ = 9.0 Hz), 94.9 (d, $J_{C\text{-}F}$ = 192.7 Hz), 81.4 (d, $J_{C\text{-}F}$ = 16.9 Hz), 74.9 (d, $J_{C\text{-}F}$ = 24.4 Hz), 61.7; HRMS (m/z) $[M+H]^+$ calcd for $C_9H_{12}FN_6O_5$ 303.0848, found 303.0851.

*4.1.2.2. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-(methoxyamino)pyrimidin-2(1H)-one (**1b**).* The product was obtained according to General Procedure B, as a pale yellow solid (73%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.00 (dd, *J* = 8.4, 2.0 Hz, 1H), 6.38 (dd, *J* = 11.2, 5.6 Hz, 1H), 5.55 (d, *J* = 8.4 Hz, 1H), 5.13 (dt, *J* = 54.0 Hz, 5.6 Hz, 1H), 4.46 (dd, *J* = 22.8, 5.2 Hz, 1H), 3.82–3.80 (m, 2H), 3.77 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 149.4, 144.3, 131.6 (d, $J_{C\text{-}F}$ = 2.8 Hz), 96.9, 96.3 (d, $J_{C\text{-}F}$ = 8.8 Hz), 94.9 (d, $J_{C\text{-}F}$ = 192.7 Hz), 81.4 (d, $J_{C\text{-}F}$ = 17.0 Hz), 74.9 (d, $J_{C\text{-}F}$ = 24.4 Hz), 61.7, 60.5; HRMS (m/z) $[M+H]^+$ calcd for $C_{10}H_{14}FN_6O_5$ 317.1004, found 317.1008.

*4.1.2.3. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-(ethoxyamino)pyrimidin-2(1H)-one (**1c**).* The product was obtained according to General Procedure B, as a white solid (75%); ^{1}H NMR (400 MHz, CD_3OD) δ 6.99 (dd, *J* = 8.4, 1.2 Hz, 1H), 6.39 (dd, *J* = 11.2, 5.6 Hz, 1H), 5.57 (d, *J* = 8.4 Hz, 1H), 5.13 (dt, *J* = 54.4, 5.6 Hz, 1H), 4.47 (dd, *J* = 22.8, 5.2 Hz, 1H), 4.01 (q, *J* = 7.2 Hz, 2H), 3.85–3.77 (m, 2H), 1.25 (t, *J* = 7.2 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 149.4, 144.2, 131.4 (d, $J_{C\text{-}F}$ = 2.7 Hz), 97.1, 96.3 (d, $J_{C\text{-}F}$ = 8.9 Hz), 94.9 (d, $J_{C\text{-}F}$ = 192.7 Hz), 81.4 (d, $J_{C\text{-}F}$ = 16.9 Hz), 74.9 (d, $J_{C\text{-}F}$ = 24.4 Hz), 68.9, 61.7, 13.4; HRMS (m/z) $[M+H]^+$ calcd for $C_{11}H_{16}FN_6O_5$ 331.1161, found 331.1167.

*4.1.2.4. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-(methoxy(methyl)amino)pyrimidin-2(1H)-one (**1d**).* The product was obtained according to General Procedure A, as a white solid (70%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.88 (dd, *J* = 7.6, 1.2 Hz, 1H), 6.46 (dd, *J* = 12.4, 5.2 Hz, 1H), 6.31 (d, *J* = 7.6 Hz, 1H), 5.20 (dt, *J* = 53.6, 4.8 Hz, 1H), 4.47 (dd, *J* = 21.6, 4.4 Hz, 1H), 3.83 (s, 2H), 3.75 (s, 3H), 3.36 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.8, 155.9, 142.3 (d, $J_{C\text{-}F}$ = 1.7 Hz), 97.3 (d, $J_{C\text{-}F}$ = 7.5 Hz), 94.6 (d, $J_{C\text{-}F}$ = 192.5 Hz), 91.4, 83.5 (d, $J_{C\text{-}F}$ = 17.1 Hz), 75.0 (d, $J_{C\text{-}F}$ = 25.1 Hz), 61.9, 60.2, 32.9; HRMS (m/z) $[M+H]^+$ calcd for $C_{11}H_{16}FN_6O_5$ 331.1161, found 331.1166.

*4.1.2.5. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-((2-hydroxyethyl)amino)pyrimidin-2(1H)-one (**1e**).* The product was obtained according to General Procedure A, as a white solid (39%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.64 (dd, *J* = 7.6, 1.2 Hz, 1H), 6.47 (dd, *J* = 12.4, 5.2 Hz, 1H), 5.89 (d, *J* = 7.6 Hz, 1H), 5.17 (dt, *J* = 54.0, 4.8 Hz, 1H), 4.45 (dd, *J* = 22.0, 4.8 Hz, 1H), 3.82 (s, 2H), 3.67 (t, *J* = 5.6 Hz, 2H), 3.49 (t, *J* = 5.6 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.3, 156.7, 140.0 (d, $J_{C\text{-}F}$ = 1.7 Hz), 97.0 (d, $J_{C\text{-}F}$ = 7.7 Hz), 95.6, 94.7 (d, $J_{C\text{-}F}$ = 192.6 Hz), 83.2 (d, $J_{C\text{-}F}$ = 17.0 Hz), 75.0 (d, $J_{C\text{-}F}$ = 25.0 Hz), 61.9, 59.9, 42.8; HRMS (m/z) $[M+H]^+$ calcd for $C_{11}H_{16}FN_6O_5$ 331.1161, found 331.1179.

*4.1.2.6. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-((3-hydroxypropyl)amino)pyrimidin-2(1H)-one (**1f**).* The product was obtained according to General Procedure A, as a pale yellow semisolid (65%). ^{1}H NMR (400 MHz, CD_3OD) δ 7.63 (dd, *J* = 7.6, 1.2 Hz, 1H), 6.47 (dd, *J* = 12.4, 5.2 Hz, 1H), 5.85 (d, *J* = 7.6 Hz, 1H), 5.17 (dt, *J* = 53.6, 4.8 Hz, 1H), 4.45 (dd, *J* = 21.6, 4.4 Hz, 1H), 3.82 (s, 2H), 3.59 (t, *J* = 6.4 Hz, 2H), 3.46 (t, *J* = 6.8 Hz, 2H), 1.77 (quint, *J* = 6.8 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.2, 156.7, 139.9 (d, $J_{C\text{-}F}$ = 1.7 Hz), 97.0 (d, $J_{C\text{-}F}$ = 7.7 Hz), 95.5, 94.7 (d, $J_{C\text{-}F}$ = 192.3 Hz), 83.2 (d, $J_{C\text{-}F}$ = 17.1 Hz), 75.0 (d, $J_{C\text{-}F}$ = 25.0 Hz), 61.9, 58.7, 37.1, 31.5; HRMS (m/z) $[M+H]^+$ calcd for $C_{12}H_{18}FN_6O_5$ 345.1317, found 345.1324.

*4.1.2.7. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-(bis(2-hydroxyethyl)amino)pyrimidin-2(1H)-one (**1g**).* The product was obtained according to General Procedure B, as a white solid (68%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.78 (dd, *J* = 8.0, 1.2 Hz, 1H), 6.47 (dd, *J* = 12.4, 5.2 Hz, 1H), 6.24 (d, *J* = 7.6 Hz, 1H), 5.19 (dt, *J* = 54.0, 4.8 Hz, 1H), 4.47 (dd, *J* = 21.6, 4.4 Hz, 1H), 3.82 (s, 2H), 3.79 (s, 4H), 3.73 (t, *J* = 5.6 Hz, 2H), 3.64 (t, *J* = 5.6 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 163.9, 156.0, 141.0 (d, $J_{C\text{-}F}$ = 1.6 Hz), 97.1 (d, $J_{C\text{-}F}$ = 7.7 Hz), 94.6 (d, $J_{C\text{-}F}$ = 192.4 Hz), 93.2, 83.3 (d, $J_{C\text{-}F}$ = 17.0 Hz), 75.0 (d, $J_{C\text{-}F}$ = 25.0 Hz), 61.9, 59.4, 59.2, 52.1, 51.1; HRMS (m/z) $[M+H]^+$ calcd for $C_{13}H_{20}FN_6O_6$ 375.1423, found 375.1425.

*4.1.2.8. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-morpholinopyrimidin-2(1H)-one (**1h**).* The product was obtained according to General Procedure A, as a white solid (69%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.83 (d, J = 7.6 Hz, 1H), 6.48 (dd, J = 12.4, 5.2 Hz, 1H), 6.24 (d, J = 8.0 Hz, 1H), 5.20 (dt, J = 53.6, 4.8 Hz, 1H), 4.47 (dd, J = 21.6, 4.4 Hz, 1H), 3.77 (m, 10H); ^{13}C NMR (100 MHz, CD_3OD) δ 163.0, 156.2, 141.7 (d, $J_{C\text{-}F}$ = 1.2 Hz), 97.2 (d, $J_{C\text{-}F}$ = 7.8 Hz), 94.7 (d, $J_{C\text{-}F}$ = 192.4 Hz), 91.9, 83.4 (d, $J_{C\text{-}F}$ = 17.0 Hz), 75.0 (d, $J_{C\text{-}F}$ = 25.0 Hz), 66.1, 61.9; HRMS (m/z) $[M+H]^+$ calcd for $C_{13}H_{18}FN_6O_5$ 357.1317, found 357.1323.

*4.1.2.9. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-((4-hydroxyphenethyl)amino)pyrimidin-2(1H)-one (**1i**).* The product was obtained according to General Procedure A, as a white solid (65%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.61 (d, J = 7.6 Hz, 1H), 7.03 (d, J = 8.4 Hz, 2H), 6.69 (d, J = 8.8 Hz, 2H), 6.47 (dd, J = 12.4, 5.2 Hz, 1H), 5.81 (d, J = 7.6 Hz, 1H), 5.18 (dt, J = 53.6, 4.8 Hz, 1H), 4.45 (dd, J = 21.6, 4.0 Hz, 1H), 3.81 (s, 2H), 3.55 (t, J = 7.6 Hz, 2H), 2.76 (t, J = 7.6 Hz, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 163.9, 156.8, 155.5, 139.8, 129.7, 129.3, 114.8, 97.0 (d, $J_{C\text{-}F}$ = 7.7 Hz), 95.6, 94.7 (d, $J_{C\text{-}F}$ = 192.3 Hz), 83.3 (d, $J_{C\text{-}F}$ = 17.2 Hz), 75.1 (d, $J_{C\text{-}F}$ = 25.1 Hz), 61.9, 42.0, 33.8; HRMS (m/z) $[M+H]^+$ calcd for $C_{17}H_{19}FN_6O_5$ 407.1474, found 407.1475.

*4.1.2.10. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-((2,4-dimethoxyphenyl)amino) pyrimidin-2(1H)-one (**1j**).* The product was obtained according to General Procedure A, as a white solid (56%); ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.02 (s, 1H), 7.76 (s, 1H), 7.64 (d, J = 7.2 Hz, 1H), 6.62 (d, J = 2.0 Hz, 1H), 6.50 (dd, J = 8.8, 2.4 Hz, 1H), 6.42–6.35 (m, 2H), 6.19 (s, 1H), 5.68 (s, 1H), 5.17 (dt, J = 54.0, 5.2 Hz, 1H), 4.42 (d, J = 22.4 Hz, 1H), 3.78–3.72 (m, 8H); ^{13}C NMR (100 MHz, DMSO-d_6) δ 162.5, 157.4, 154.4, 152.5, 141.1, 125.8, 119.7, 104.1, 98.8, 96.9 (d, $J_{C\text{-}F}$ = 8.4 Hz), 95.7, 94.5 (d, $J_{C\text{-}F}$ = 191.2 Hz), 82.2 (d, $J_{C\text{-}F}$ = 16.8 Hz), 74.9 (d, $J_{C\text{-}F}$ = 24.1 Hz), 62.3, 55.7, 55.4; HRMS (m/z) $[M+H]^+$ calcd for $C_{17}H_{19}FN_6O_6$ 423.1423, found 423.1426.

*4.1.2.11. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-((furan-2-ylmethyl)amino)pyrimidin-2(1H)-one (**1k**).* The product was obtained according to General Procedure A, as a pale yellow solid (67%); ^{1}H NMR (400 MHz, CD_3OD) δ 7.67 (dd, J = 7.6, 0.8 Hz, 1H), 7.41 (d, J = 0.8 Hz, 1H), 6.48 (dd, J = 12.4, 5.2 Hz, 1H), 6.34–6.30 (m, 2H), 5.88 (d, J = 7.6 Hz, 1H), 5.18 (dt, J = 53.6, 4.8 Hz, 1H), 4.56 (s, 2H), 4.46 (dd, J = 21.6, 4.4 Hz, 1H), 3.82 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 163.8, 156.7, 151.0, 142.1, 140.3 (d, $J_{C\text{-}F}$ = 1.6 Hz), 110.0, 107.3, 97.1 (d, $J_{C\text{-}F}$ = 7.7 Hz), 95.4, 94.7 (d, $J_{C\text{-}F}$ = 192.4 Hz), 83.3 (d, $J_{C\text{-}F}$ = 17.0 Hz), 75.0 (d, $J_{C\text{-}F}$ = 25.0 Hz), 61.9, 36.9; HRMS (m/z) $[M+H]^+$ calcd for $C_{14}H_{15}FN_6O_5$ 367.1161, found 367.1164.

*4.1.2.12. 1-((2R,3S,4R,5R)-5-azido-3-fluoro-4-hydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-4-((pyridin-3-ylmethyl)amino)pyrimidin-2(1H)-one (**1l**).* The product was obtained according to General Procedure A, as a white solid (68%); ^{1}H NMR (400 MHz, CD_3OD) δ 8.52 (s, 1H), 8.41 (d, J = 4.8 Hz, 1H), 7.83 (d, J = 8.0 Hz, 1H), 7.71 (d, J = 7.6 Hz, 1H), 7.40–7.36 (m, 1H), 6.47 (dd, J = 12.4, 5.2 Hz, 1H), 5.91 (dd, J = 7.6, 1.6 Hz, 1H), 5.18 (dt, J = 53.6, 4.8 Hz, 1H), 4.63 (s, 2H), 4.46 (dd, J = 21.6, 4.4 Hz, 1H), 3.82 (s, 2H); ^{13}C NMR (100 MHz, CD_3OD) δ 164.1, 156.7, 148.2, 147.5, 140.6 (d, $J_{C\text{-}F}$ = 1.5 Hz), 136.5, 134.9, 123.8, 97.1 (d, $J_{C\text{-}F}$ = 7.5 Hz), 95.3, 94.7 (d, $J_{C\text{-}F}$ = 192.3 Hz), 83.4 (d, $J_{C\text{-}F}$ = 16.9 Hz), 75.0 (d, $J_{C\text{-}F}$ = 25.0 Hz), 61.9, 41.1; HRMS (m/z) $[M+H]^+$ calcd for $C_{15}H_{16}FN_7O_4$ 378.1321, found 378.1322.

4.2. Cytotoxicity assay

To determine the cell viability, an MTT-based assay was performed [19]. Briefly, HepG2.2.15 cells were seeded at a density of 2×10^4 cells per well on 96-well plates. After incubation for 24 h, the cells were treated with medium containing the test compound of different concentrations (2, 10, 50, 250, 1250 μM). The culture medium was replaced with a fresh one on day 3 or day 6 during the 9-day experiment. On day 9, 20 μL of MTT solution (5 mg/mL) was added to each well. After incubating routinely for 4 h, the supernatants were discarded and 180 μL of DMSO was added into each well to solubilize the formazan. The absorbance (A) at 490 nm was measured by using an automatic plate reader (BIO-RAD, 168-1000XC, USA). The survival rate of HepG2.2.15 cells (%) was calculated as [1 − (A490 of experimental group/A490 of negative control) × 100%] [20].

4.3. Assessment of Anti-HBV activity screening

The HepG2.2.15 cells were plated on 24-well plates at a density of 1×10^5 cells per well and routinely cultured for 24 h. Then, the cells were treated with freshly prepared medium containing the test compound at the concentration of 1 μM or ETV as a positive control at 1 μM. Normal control cells were treated with minimum essential medium (MEM) only. Media were changed every 3 days, and the supernatants were collected on day 9. HBV DNA was extracted by a viral DNA extraction kit according to the manufacturer's instructions and the viral DNA levels were detected by an FQ- PCR detection kit according to the manufacturer's protocol. Amplification and detection were performed on ABI7500 fluorescent quantitative PCR instrument. The program was optimized as follows: 37 °C for 5 min, at 94 °C for 2 min (denaturation), followed by 40 cycles of amplification (at 94 °C for 20 s, 55 °C for 30 s, 72 °C for 10 s). Inhibition rate of HBV DNA (%) = [1 − (HBV DNA concentration of experimental group/HBV DNA concentration of negative control) × 100%].

4.4. Assessment of inhibitory activity on the HBsAg and HBeAg secretion

The HepG2.2.15 cells were seeded at a density of 2×10^4 per well on 24-well plates and routinely cultured for 24 h. Then, the culture medium was replaced with freshly prepared medium containing the test compound at various concentrations (1, 0.1, 0.01 μM) or lamivudine (3 TC, 25 μM as positive control) in triplicate. Negative control groups were treated with RPMI 1640 only. Media were changed on day 3, 6 and 9, and the supernatants were collected. The levels of HBsAg in the supernatants were detected by the ELISA kit (Kehua Bio-engineering Corporation, Shanghai, PR China) according to the manufacturer's protocol. The absorbance (A) at 450/630 nm was measured by using an automatic plate reader. Inhibition rate of HBsAg (%) = [1-(A450/630 of experimental group/A450/630 of negative control) × 100%] [13].

4.5. Assessment of inhibitory effect on HBV DNA replication

The HepG2.2.15 cells were plated at a density of 2×10^4 cells per well on 24-well plates and routinely cultured for 24 h. Then, the cells were treated with freshly prepared medium containing compound **1g** (1, 0.1, 0.01 μM) or lamivudine (3 TC, 436 μM as positive control). Normal control cells were treated with RPMI 1640 only. Media were changed every 3 days, and both the supernatants and cells were collected. The extracellular and intracellular HBV DNA was extracted by viral DNA extraction kit according to the manufacturer's instructions. The concentration of extracted HBV DNA

was determined by real-time FQ-PCR, performed in Light-Cycler 1.5 (Roche, Mannheim, Germany) using the HBV fluorescent quantitative PCR detection kit (Piji Biotechnology Development, Shenzhen, PR China) according to the manufacturer's protocol. Inhibition rate of HBV DNA (%) = [1 − (HBV DNA concentration of experimental group/HBV DNA concentration of negative control) × 100%] [21,22].

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