Design, Synthesis, and Evaluation of a Set of Carboxylic Acid and Phosphate Prodrugs Derived from HBV Capsid Protein Allosteric Modulator NVR 3-778

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Abstract: Hepatitis B virus (HBV) capsid protein (Cp) is necessary for viral replication and the maintenance of viral persistence, having become an attractive target of anti-HBV drugs. To improve the water solubility of HBV capsid protein allosteric modulator (CpAM) NVR 3-778, a series of novel carboxylic acid and phosphate prodrugs were designed and synthesized using a prodrug strategy. In vitro HBV replication assay showed that these prodrugs maintained favorable antiviral potency (EC50 = 0.28–0.42 µM), which was comparable to that of NVR 3-778 (EC50 = 0.38 µM). More importantly, the cytotoxicity of prodrug N8 (CC50 > 256 µM) was significantly reduced compared to NVR 3-778 (CC50 = 13.65 ± 0.21 µM). In addition, the water solubility of prodrug N6 was hundreds of times better than that of NVR 3-778 in three phosphate buffers with various pH levels (2.0, 7.0, 7.4). In addition, N6 demonstrated excellent plasma and blood stability in vitro and good pharmacokinetic properties in rats. Finally, the hemisuccinate prodrug N6 significantly improved the candidate drug NVR 3-778’s water solubility and increased metabolic stability while maintaining its antiviral efficacy.

Keywords: HBV; capsid; CpAM; NVR 3-778; prodrug strategy; water solubility

1. Introduction

Chronic hepatitis B is an infectious disease characterized by liver damage caused by persistent infection with hepatitis B virus (HBV). Further deterioration of hepatitis B will cause a series of complications, such as disorders of liver metabolism, liver failure, cirrhosis, and liver cancer [1]. According to the World Health Organization, about 296 million people worldwide suffer from chronic HBV infection, accounting for about 4% of the world’s population, and about 820,000 people die of chronic viral-hepatitis-related liver disease every year [2]. Hepatitis B is highly contagious and difficult to cure, which seriously endangers human public health and social development. Therefore, it is urgent to develop safe and effective anti-HBV drugs.

At present, interferons and nucleos(t)ide analogues are used to treat HBV but neither can achieve elimination of HBV. The goal in the treatment of hepatitis B is to maximize the inhibition of HBV replication, reduce the HBV antigen levels, relieve the symptoms of hepatitis and liver fibrosis, reduce and delay the occurrence of complications, thereby improving the liver function and quality of life of patients [3]. Clinically, long-term use of the current drugs accelerates the occurrence of drug resistance or adverse reactions. In addition, nucleos(t)ide analogues have to be used for a prolonged period or possibly life-long to continuously inhibit HBV replication [4–6]. Therefore, it becomes a hot area...
of research in anti-HBV drugs that use new strategies of medicinal chemistry to develop non-nucleoside HBV inhibitors with novel mechanisms of action.

HBV capsid protein (Cp) plays a key role in multiple stages of HBV replication, including subcellular transport, cccDNA maintenance, capsid assembly, and the following processes of pregeomic RNA encapsidation and viral DNA synthesis [7–9]. Therefore, HBV Cp has become an attractive target for mechanism-oriented antiviral therapy due to the important role in viral replication. Capsid protein allosteric modulators (CpAMs) disrupt the functional capsid assembly by directly targeting Cp, thereby inhibiting HBV replication [10]. The CpAMs reported so far include heteroaryl-dihydropyrimidines (HAPs), sulfamoylbenzamides (SBAs), and phenylacrylamides (PPAs) [11–13]. The representative compounds in different structural types are shown in Figure 1.

![Figure 1. The structures of some representative HBV CpAMs.](image)

NVR 3-778 (2) is an SBAs-CpAM that was demonstrated to have antiviral activity in HBV-infected patients [11]. NVR 3-778 showed significant antiviral activity in the HepG2.2.15 cell line (EC_{50} = 0.40 \, \mu M). It has synergistic antiviral effects with nucleoside inhibitors in vitro [14] and with polyethylene glycol interferon in clinical trials [15]. However, the relatively low water solubility (0.003 mg/mL at pH = 2.0) could limit its clinical application, and its development was stopped because of a limited efficacy at clinically feasible doses [16].

Many drugs with excellent pharmacological activity often had defects in physical characteristics and pharmacokinetics, such as poor water solubility, low oral bioavailability, and rapid metabolism, which limited the direct clinical application [17–19]. Both carboxylic acid and phosphate are charged groups, and the introduction of such groups can improve the water solubility of compounds. For example, after the introduction of carboxylic acid, the water solubility of adrenocorticotropic hormone prednisolone was improved and can be used for injection. In addition, when the antifungal drug fluconazole was introduced to the phosphate, its water solubility was improved and the dosage was reduced [20–22]. Consequently, in this study, to improve the water solubility and reduce the toxicity of NVR 3-778, we designed and synthesized a series of carboxylic acid and phosphate prodrugs.

Herein, four carboxylic acid and phosphate compounds were tested for anti-HBV activity in vitro. Among them, N6 was selected for further water solubility testing, plasma and whole blood stability experiments, and preliminary pharmacokinetic evaluation in rats. These experimental results will be discussed in detail.

2. Results and Discussion
2.1. Synthesis of the Target Molecules

As shown in Scheme 1, 3-chlorosulfonyl-4-fluorobenzoic acid (N1) was reacted with 4-hydroxyxipiperidine to obtain intermediate N2 by nucleophlic sulfonyl substitution reaction. N2 was treated with 3,4,5-trifluoroaniline to yield NVR 3-778 (N3) by the amide condensation reaction [23]. Next, N3 reacted with various anhydrides to give end-product prodrugs N6–N8 under DMAP catalysis. In addition, N3 and dibenzyl disopropylphosphoramidite reacted in the presence of 1H-imidazole and 3-chlorobenzoic acid to obtain
N4. Subsequently, the benzyl group was removed from N4 under the action of hydrogen and palladium carbon to obtain phosphate prodrug N5 [24].

Scheme 1. Reagents and conditions. (i) 4-hydroxypiperidine, DIEA, MeCN, 0 °C, 8 h; (ii) 3,4,5-trifluoroaniline, HATU, DIEA, CH3Cl2, r.t., 8 h; (iii) 1H-imidazole, dibenzyl disopropylphosphoramidite, 3-chlorobenzoic acid, DCM, 0 °C, 10 h; (iv) H2, palladium carbon, THF, r.t., 12 h; (v) various anhydrides, DMAP, Et3N, CH2Cl2, r.t., 10 h.

2.2. Biological Activity

The target compounds N5-N8 were evaluated for their antiviral potency against HBV replication, as well as cytotoxicity in Hep38.7-Tet cells, which reproduces HBV replication from a chromosome-integrated HBV transgene under depletion of tetracycline from the medium [25]. The 50% effective concentration (EC50) for anti-HBV activity, 50% cytotoxic concentration (CC50), and selectivity index (SI) calculated by CC50/EC50 ratio are shown in Table 1. The antiviral activity of all prodrugs (EC50 = 0.42–0.28 µM) was similar to the NVR 3-778 (EC50 = 0.38 ± 0.047 µM), but the toxicity of compound N8 (CC50 > 256 µM, SI > 731) was reduced by at least 18-fold, compared with NVR 3-778 (CC50 = 13.65 ± 0.21 µM, SI = 36). When R was replaced by phosphate and 4-oxo-butanolic acid, compound N5 (EC50 = 0.42 ± 0.069 µM, 13.66 ± 1.45 µM) and N6 (EC50 = 0.35 ± 0.048 µM, CC50 = 12.62 ± 1.05 µM) had similar antiviral activity and toxicity with NVR 3-778 (EC50 = 0.38 ± 0.047 µM, 12.65 ± 0.21 µM). The antiviral activity of N7 (EC50 = 0.28 ± 0.024 µM, CC50 = 12.98 ± 1.74 µM) was slightly better than that of NVR 3-788 when R was replaced by 5-oxo-butanolic acid. When R was 6-oxo-hexanoic acid, prodrug N8 (EC50 = 0.35 ± 0.032 µM, CC50 > 256 µM) not only maintained the antiviral activity, but also greatly reduced the cytotoxicity.

Table 1. Anti-HBV activity and cytotoxicity of the newly synthesized compounds.

| Compds | R       | EC50 (µM) a | CC50 (µM) b | SI c  |
|--------|---------|-------------|-------------|-------|
| N5     | -OH     | 0.42 ± 0.069| 13.66 ± 1.45| 33    |
| N6     | -OH     | 0.35 ± 0.048| 12.62 ± 1.05| 36    |
| N7     | -OH     | 0.28 ± 0.024| 12.98 ± 1.74| 46    |
| N8     | -OH     | 0.35 ± 0.032| >256        | >731  |
| NVR 3-778 | -OH | 0.38 ± 0.047| 13.65 ± 0.21| 36    |

a EC50: the concentration of 50% inhibition for HBV DNA in Hep38.7-Tet cells supernatant. b CC50: the concentration that kills 50% of Hep38.7-Tet cells, as determined using the WST method. c SI: selectivity index, the ratio of CC50/EC50.
2.3. Water Solubility

In order to verify whether the prodrug strategy was successful in improving the water solubility of NVR 3-778, representative compound N6 was selected to test water solubility under three different pH. As shown in Table 2, compared with NVR 3-778, the water solubility of N6 was more than 180 times at pH = 2 (N6: 695 µg/mL, NVR 3-778: 3.82 µg/mL), 310 times at pH = 7 (N6: 7500 µg/mL, NVR 3-778: 24.02 µg/mL), and 900 times at pH = 7.4 (N6: 4416 µg/mL, NVR 3-778: 4.84 µg/mL). The experimental data confirmed that the introduction of carboxylic acid markedly improved the solubility of NVR 3-778. The experimental results verified the rationality of prodrug strategy, and the improvement of water solubility is of great significance for the development of new pharmaceutical dosage forms.

Table 2. Water solubility of N6 and NVR 3-778 at different pH.

| Compds   | pH = 2.0 (µg/mL) | pH = 7.0 (µg/mL) | pH = 7.4 (µg/mL) |
|----------|------------------|------------------|------------------|
| NVR 3-778| 3.82             | 24.02            | 4.84             |
| N6       | 695              | 7500             | 4416             |

2.4. Plasma and Whole Blood Stability

In order to verify the stability of prodrug in vitro, the stability of NVR 3-778 and prodrug N6 in plasma at 4 °C and whole blood at 37 °C was tested by the LC-MS/MS and characterization of their stability by peak area. As shown in Table 3, two hours later, the residual amounts of NVR 3-778 in plasma and whole blood were 85% and 91%, respectively, and only about 10% of drugs were metabolized after 2 h of storage. Prodrug N6 was more stable, and the residual amounts of N6 in plasma and whole blood were close to 100%, which means almost no metabolic degradation occurred after two hours of storage. The results showed that the introduction of carboxylic acid appropriately enhanced the metabolic stability of NVR 3-778 in vitro.

Table 3. Stability of NVR 3-778 and N6 in plasma at 4 °C and whole blood at 37 °C.

| Compds   | Plasma (4 °C) | Whole Blood (37 °C) |
|----------|--------------|---------------------|
|          | 0 h          | 2 h Residual | 0 h          | 2 h Residual |
| N6       | 0.88 ± 0.052 | 0.89 ± 0.016 | 101%        | 0.65 ± 0.048 | 0.70 ± 0.026 | 108%      |
| NVR 3-778| 0.12 ± 0.0078| 0.10 ± 0.0066 | 85%         | 0.18 ± 0.016 | 0.16 ± 0.0026 | 91%       |

2.5. Pharmacokinetic Experiments of N6

In order to explore the metabolism and bioavailability of prodrug in vivo after oral administration, the main pharmacokinetic parameters were tested. After oral administration of N6 and NVR 3-778 to the two groups of rats, we detected their plasma concentration, respectively. As shown in Figure 2, after oral administration of N6, the concentration of N6 in plasma decreased rapidly, and N6 could not be detected after 2 h, while the concentration of NVR 3-778 in plasma increased rapidly and reached the peak at 1 h. This indicated that N6 was completely converted into NVR 3-778 within 2 h in rats. As shown in Table 4, compared with direct oral administration of NVR 3-778, oral prodrug N6 (t1/2 = 1.92 ± 0.227 h) can increase the half-life of NVR 3-778 (t1/2 = 1.40 ± 0.250 h). In addition, N6 (Cmax = 747 ± 104 ng/mL) reduced the maximum concentration of the drug in the body compared with NVR 3-778 (Cmax = 1161 ± 261 ng/mL), thereby appropriately reducing the side effects and irritation of drugs. Considering the different molar doses (N6: 18.8 nmol/kg; NVR 3-778: 23.1 nmol/kg), N6 (AUC0-Inf = 2222 ± 935 ng.h/mL) had a similar drug–time curve area as NVR 3-778 (AUC0-Inf = 3937 ± 948 ng.h/mL), suggesting that N6 and NVR 3-778 had analogous oral bioavailability. The experimental results confirmed that N6 had longer half-life and lower maximum concentration in vivo than NVR 3-778. In
addition, N6 was completely transformed into NVR 3-778 in vivo, and both had favorable bioavailability. Compared with NVR3-778, N6 can release drugs more slowly, which can improve the narrow therapeutic window of NVR3-778 to some extent.

Figure 2. Plasma drug concentration–time curve of N6 and NVR 3-778 after oral administration.

Table 4. Main pharmacokinetic parameters after oral administration of N6 and NVR 3-778.

| PK Parameters | N6 (18.8 nmol/kg) | NVR 3-778 (23.1 nmol/kg) |
|---------------|-------------------|--------------------------|
| T1/2 (h) a | ND b | 1.92 ± 0.227 | 1.40 ± 0.250 |
| Tmax (h) c | 0.25 | 1.00 ± 0.00 | 0.833 ± 0.289 |
| Tlast (h) d | 1.00 | 10.0 | 10.0 |
| Cmax (ng/mL) e | 14.7 ± 1.31 | 747 ± 104 | 1161 ± 261 |
| AUC0-last (ng.h/mL) f | 8.13 ± 1.50 | 2153 ± 877 | 3893 ± 948 |
| AUC0-inf (ng.h/mL) f | ND b | 2222 ± 935 | 3937 ± 948 |
| MRT0-last (h) g | 0.487 | 2.67 ± 0.489 | 2.67 ± 0.106 |
| MRT0-inf (h) g | ND b | 2.95 ± 0.610 | 2.78 ± 0.144 |

a T1/2: the time required for concentration of drug in blood to decrease by half. b ND: not determined. c Tmax: time required to reach peak drug concentration after administration. d Tlast: time of the last point. e Cmax: the highest blood concentration after administration. f AUC: the area enclosed by the plasma concentration curve versus the time axis. g MRT: the average residence time of drug molecules in the body. 0-last: from the first time point of oral administration to the last time point. 0-inf: from the first time point of oral administration to the time point at theoretical infinity.

3. Conclusions

To improve the water solubility and safety index of HBV CpAM NVR 3-778, we designed and synthesized a series of carboxylic acid and phosphate prodrugs. The antiviral activity of all prodrugs was similar to NVR 3-778. Compared with NVR 3-778, the cytotoxicity of compound N8 was significantly reduced (CC50 > 256 µM, SI > 731). In three different pH phosphate buffer solutions (2.0, 7.0, and 7.4), the solubility of prodrug N6 was hundreds of times better than that of NVR 3-778, which is of great significance for
the development of new preparation types. In addition, N6 exhibited excellent stability in plasma and whole blood, which provided the basis for further evaluation of druggability in vivo. Pharmacokinetics in rats showed that prodrug N6 and NVR 3-778 had similar bioavailability. What is more, N6 can be gradually metabolized to NVR 3-778, which reduces irritation by reducing the maximum drug concentration in vivo. In conclusion, the prodrug N6 successfully improved the water solubility of the candidate drug NVR 3-778. While maintaining the curative effect, prodrug N6 improved metabolic stability moderately, indicating that it has a good development prospect.

4. Experimental Section

4.1. Chemistry

$^1$H NMR and $^{13}$C NMR spectra were recorded on Bruker Avance-600 NMR spectrometer with DMSO-$d_6$ as the solvent. Chemical shifts were expressed in $\delta$ values (ppm), and $J$ values were expressed in hertz (Hz). Various solvents were obtained from Sinopharm Chemical Reagent Co., Ltd. (SCRC, Shanghai, China), which were of AR grade. Reagents were purchased from Bide Pharmatech Co., Ltd. TLC was performed on silica gel GF254 (Merck) and irradiated by ultraviolet light ($\lambda = 254$ nm). Flash column chromatography was performed on a column packed with Silica Gel60 (200–300 mesh). Spectra of all compounds are in Supplementary Material.

4.1.1. 4-Fluoro-3-((4-hydroxypiperidin-1-yl)sulfonyl)benzoic Acid (N2)

4-hydroxypiperidine (10.00 mmol, 1.01 g) was dissolved in 10 mL anhydrous acetonitrile, and $N,N$-diisopropylethylamine (20.00 mmol, 3.48 mL) was added. At 0 °C, 5 mL acetonitrile solution containing 3-chlorosulfonyl-4-fluorobenzoic acid (10.00 mmol, 2.38 g) was slowly dropped and stirred for 8 h. After the reaction was completed, 10 mL 1N hydrochloric acid solution was added after the solvent was evaporated under reduced pressure. Then, it was extracted with ethyl acetate (3 × 8 mL), combining organic phase, and washed with saturated sodium chloride solution (3 × 15 mL). The solvent was dried by anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to obtain N2. White solid, yield: 60.0%. EI-MS: 302.5 [M − H]$^-$, C$_{12}$H$_{14}$FNO$_5$S (303.30).

4.1.2. 4-Fluoro-3-((4-hydroxypiperidin-1-yl)sulfonyl)-N-(3,4,5-trifluorophenyl)benzamide (N3)

N2 (6.00 mmol, 1.82 g), O-(7-azabenzotriazol-1-yl)-N,N,N',N'-tetramethylurea hexafluorophosphate (9.00 mmol, 3.43 g) was dissolved in 15 mL dichloromethane and stirred at room temperature for 1 h. Then, N,N-diisopropylethylamine (18.00 mmol, 3.13 mL) and 3,4,5-trifluoroaniline (6.00 mmol, 0.88 g) were added and stirred at room temperature for 7 h. After the reaction completed, the solvent was evaporated under reduced pressure, then 20 mL saturated sodium bicarbonate solution was added to the residue in the bottle. It was extracted with ethyl acetate (3 × 10 mL), and the organic layers were combined and washed with saturated sodium chloride solution (3 × 30 mL). The organic phases were dried over anhydrous sodium sulfate, filtered, and the solvent was evaporated to dryness under reduced pressure. White solid, yield: 34.0%. EI-MS: 432.5 [M + H]$^+$, C$_{18}$H$_{16}$F$_4$N$_2$O$_4$S (432.39).

4.1.3. Dibenzyl (1-((2-Fluoro-5-((3,4,5-trifluorophenyl)carbamoyl)phenyl)sulfonyl)piperidin-4-yl) Phosphate (N4)

N3 (2.30 mmol, 1.00 g), 1H-imidazole (3.50 mmol, 0.25 g), and dibenzyl diisopropylphosphoramidite (4.60 mmol, 1.56 mL) were dissolved in 20 mL dichloromethane and stirred for four hours, then 3-chlorobenzoic acid (4.60 mmol, 0.79 g) was added at 0 °C and stirred for an additional 6 h. After the completion of the reaction, 20 mL saturated sodium bicarbonate was added, and then dichloromethane (3 × 10 mL) was added for extraction. The organic phases were combined and washed with saturated sodium chloride (3 × 15 mL). The organic phase was dried over anhydrous sodium sulfate and subjected
to column chromatography after stirring with silica gel. White solid, yield: 30.0%. EI-MS: 693.06 [M + H]+, C32H22F4N2O2PS (692.14).

4.1.4. 1-((2-Fluoro-5-((3,4,5-trifluorophenyl)carbamoyl)phenyl)sulfonyl)piperidin-4-yl Dihydrogen Phosphate (N5)

N4 (0.20 g) and Palladium carbon (0.02 g) were dissolved in 15 mL tetrahydrofuran and stirred under hydrogen atmosphere for 12 h. After completion of the reaction, the reaction solution was filtered through celite, and the solvent was evaporated to dryness under reduced pressure and purified by high performance liquid chromatography (HPLC).

White solid, yield: 26.4%, mp: 162–164 °C. 1H NMR (600 MHz, DMSO-d6) δ 10.79 (s, 1H), 8.33 (dd, J = 20.1, 7.3, 3.0 Hz, 2H), 7.75–7.65 (m, 3H), 4.27 (s, 2H), 4.03 (J = 7.6, 3.6 Hz, 1H), 3.15–2.96 (m, 4H), 1.92–1.64 (m, 4H). 13C NMR (151 MHz, DMSO-d6) δ 163.53, 160.89, 159.17, 149.89 (ddd, J = 15 Hz, 3 CF = 3 Hz, C × 2), 135.18 (m), 130.98 (J = 7.7, 3 CF = 3 Hz), 130.40 (C × 2), 125.21 (d, J = 15 Hz), 117.98 (d, 2 CF = 22.5 Hz), 104.84 (d, 2 CF = 22.5 Hz, C × 2), 69.26, 42.19 (C × 2), 31.27 (C × 2). El-MS: 513.08 [M + H]+, C18H12F4N2O2PS (512.04).

4.1.5. General Procedure for the Synthesis of Target Compounds N6–N8

N3 (2.01 mmol, 0.87 g), triethylamine (4.02 mmol, 0.56 mL), 4-dimethylaminopyridine (4.02 mmol, 0.49 g), and different acid anhydrides (12.06 mmol) were added to 15 mL anhydrous dichloromethane and stirred at room temperature for 10 h. After the reaction was completed, 20 mL 1N hydrochloric acid solution was added to the reaction solution to quench the reaction and extracted with dichloromethane (3 × 10 mL). The organic layers were combined, washed with saturated sodium chloride solution (3 × 30 mL), and the organic phase was washed with dried-over anhydrous sodium sulfate; it was filtered, evaporated to dryness under reduced pressure, and separated by silica gel column chromatography.

4-((1-((2-fluoro-5-((3,4,5-trifluorophenyl)carbamoyl)phenyl)sulfonyl)piperidin-4-yl)oxy)-4-oxobutanoic acid (N6). White solid, yield: 43.9%, mp: 138–140 °C. 1H NMR (600 MHz, DMSO-d6) δ 12.22 (s, 1H), 10.83 (s, 1H), 8.34 (ddd, J = 15.8, 7.6, 3.3 Hz, 2H), 7.84–7.63 (m, 3H), 4.81 (tt, J = 7.7, 3.7 Hz, 1H), 3.31 (d, J = 4.6 Hz, 2H), 3.15 (ddd, J = 12.1, 7.8, 3.5 Hz, 2H), 2.44 (tt, J = 6.5, 3.3 Hz, 4H), 1.88 (ddd, J = 11.5, 7.2, 3.7 Hz, 2H), 1.61 (ddq, J = 12.0, 7.9, 3.8 Hz, 2H). 13C NMR (150 MHz, DMSO-d6) δ 173.85, 171.79, 164.01, 160.64 (d, J = 259.7 Hz), 150.43 (ddd, J = 243.6 Hz, 3 CF = 9.9 Hz, 3 CF = 5.2 Hz, C × 2), 136.23 (ddd, J = 136.4 Hz, 2 CF = 12.1 Hz, 2 CF = 4.2 Hz, J = 2 CF = 9.7 Hz), 131.39 (d, J = 3 CF = 3.3 Hz), 130.93 (C × 2), 125.78 (d, J = 15 Hz), 118.62 (d, J = 15 Hz, C × 2), 105.34 (d, J = 24.4 Hz, C × 2), 68.28, 51.83, 43.04, 30.18, 29.44, 29.14, 28.98. EI-MS: 531.3 [M – H]–, C22H20F2N2O2S (532.46).

6-((1-((2-fluoro-5-((3,4,5-trifluorophenyl)carbamoyl)phenyl)sulfonyl)piperidin-4-yl)oxy)-6-oxohexanoic acid (N7). White solid, yield: 42.6%, mp: 135–137 °C. 1H NMR (600 MHz, DMSO-d6) δ 11.24 (s, 1H), 9.97 (s, 1H), 8.25 (ddd, J = 16.8, 8.6, 3.8 Hz, 2H), 6.90–6.73 (m, 3H), 4.00 (tt, J = 7.8, 3.8 Hz, 1H), 2.74–2.50 (m, 4H), 1.69 (p, J = 1.8 Hz, 2H), 1.64–1.12 (m, 4H), 1.15–0.70 (m, 4H). 13C NMR (150 MHz, DMSO-d6) δ 174.33, 172.20, 164.01, 160.50 (d, J = 259.7 Hz), 150 (ddd, J = 244.6 Hz, 3 CF = 9 Hz, 3 CF = 4.5 Hz, C × 2), 135.7 (m), 134.81 (d, J = 3 CF = 16.5 Hz), 131.43 (d, J = 3 CF = 3.0 Hz), 130.88 (C × 2), 125.95 (d, J = 150.0 Hz, 2 CF = 22.65 Hz), 118.65 (d, J = 2 CF = 22.65 Hz, C × 2), 105.39 (d, J = 2 CF = 24.1 Hz, 3 CF = 1.9 Hz), 68.31, 43.16, 33.33, 33.28, 33.10, 30.31, 20.47, 20.37. EI-MS: 545.3 [M – H]–, C22H20F2N2O2S (546.11).

6-((1-((2-fluoro-5-((3,4,5-trifluorophenyl)carbamoyl)phenyl)sulfonyl)piperidin-4-yl)oxy)-6-oxohexanoic acid (N8). White solid, yield: 39.6%, mp: 132–134 °C. 1H NMR (600 MHz, DMSO-d6) δ 11.16 (s, 1H), 9.97 (s, 1H), 7.97–7.31 (m, 2H), 7.27–7.51 (m, 3H), 4.00 (tt, J = 7.6, 3.8 Hz, 1H), 2.56 (ddd, J = 11.5, 6.8, 4.0 Hz, 4H), 2.33 (ddd, J = 12.2, 8.4, 3.5 Hz, 2H), 1.70 (p, J = 1.9 Hz, 2H), 1.52–1.31 (m, 2H), 1.26–0.97 (m, 2H), 0.96–0.70 (m, 2H), 0.68 (tttd, J = 7.7, 5.4, 4.6, 2.8 Hz, 2H). 13C NMR (150 MHz, DMSO-d6) δ 174.07, 171.85, 163.45, 160.08 (d, J = 2 CF = 259.7 Hz), 149.90 (ddd, J = 241.6 Hz, 3 CF = 10.5 Hz, 3 CF = 6.0 Hz, C × 2), 135.23 (m), 134.26 (d, J = 15.0 Hz, 130.87 (d, J = 4.5 Hz, C × 2), 125.38 (d, J = 15.1 Hz), 118.01 (d, J = 24.1 Hz).
104.83 (d, $^2J_{\text{CF}} = 25.5$ Hz, C × 2), 67.67, 42.61, 42.54, 38.15, 33.28, 33.19, 29.76, 23.85, 23.76. EI-MS: 559.27 [M − H$^-$], C$_{24}$H$_{24}$F$_4$N$_2$O$_7$S (560.12).

4.2. In Vitro Anti-HBV Assay

4.2.1. Assessment of Inhibitory Activity on HBV Replication in Hep38.7-Tet Cells

Hep38.7-Tet cells were cultured with DMEM/F-12/ GlutaMAX supplemented with 10% fetal bovine serum, and 10 mM HEPES, 200 unit/mL penicillin, 200 µg/mL streptomycin, 5 µg/mL insulin, and 400 ng/mL tetracycline were added at 37 °C in 5% CO$_2$. Tetracycline was removed when inducing HBV replication. Three days after seeding the cells, the cells were treated with or without compounds for 6 days and the culture supernatants were recovered to quantify HBV DNA using the primers 5$'$-AAGGTAGGAGCTGGAGCATTCG-3$'$, 5$'$-AGGCGGATTTGCTGGCAAAG-3$'$, and a probe 5$'$-FAM-AGCCCTCAGGTCAGGGCATAC-TAMRA-3$'$ by real-time fluorescence quantitative PCR, as described [26]. We set concentration gradients (1.00 µM, 0.33 µM, and 0.11 µM) to test and calculate EC$_{50}$ of all compounds.

4.2.2. Cytotoxicity Assay

The WST assays were performed by using Cell Counting Kit-8 (CCK-8) (DOJINDO) according to the manufacturer’s protocol. CCK-8 solution was added to the cells, which were incubated at 37 °C for 15 min and the absorbance at 450 nm was measured. CC$_{50}$ was calculated by the concentration gradient (1.0 µM, 4.0 µM, 16.0 µM, 64.0 µM, and 256.0 µM).

4.3. Water Solubility Test of N6

The compound was dissolved in DMSO to prepare the mother liquor with a concentration of 10 mg/mL, and then 10 µL mother liquor was added to 1 mL phosphate buffer with different pH (pH = 2.0, 7.0, and 7.4). The solution was oscillated for 2 h at 3000 rpm of the vortex oscillator, and the precipitation of compounds was observed visually. The standard curve can be established by increasing the concentration of mother liquor until turbidity appears. The peak area (A) of different concentrations (c) was determined by HPLC. The standard curve was established with the concentration and peak area as abscissa and ordinate, respectively, and the standard curve equation A = kc + b was calculated.

The standard curve of NVR 3-778 concentration–peak area was established by measuring the peak area at five concentrations of 1000, 200, 40, 8.0, and 1.6 µg/mL. The standard curve equation was A = 35,268c + 80,359 with R$^2$ = 0.9999. By measuring the peak area at 4000 µg/mL, 1000 µg/mL, 200 µg/mL, 40 µg/mL, and 8.0 µg/mL, the standard curve of N6 concentration–peak area was established: A = 16,303c + 714,762, R$^2$ = 0.999. Since the solubility of N6 in phosphate buffer solution (pH = 7.0, 7.4) had exceeded the detector range, the saturated solution of N6 was diluted 10 times for testing. On this basis, the saturated solution (pH = 2.0, 7.0, and 7.4) of phosphate buffer at different pH values was prepared and fully oscillated for 1 h before filtration. Finally, the absorption peak area of the sample at different pH phosphate buffer (pH = 2.0, 7.0, and 7.4) was determined (measured twice). According to the standard curve equation, the corresponding concentration was calculated, which was the solubility of the compound.

4.4. Plasma and Whole Blood Stability Experiments of N6

A total of 1 mL of rat plasma was placed in ice water at 4 °C and 1 mL of rat whole blood was placed in a water bath at 37 °C for use, respectively. DMSO solution with compound concentration of 1 mM was prepared, and then 10 µL compound solution was added to 90 µL 45% methanol aqueous solution to obtain a quantitative solution with a concentration of 100 µM. A 2 µL compound solution was added to 98 µL 4 °C plasma and 37 °C whole blood, and three portions were prepared to reach the final concentration of 2 µM, respectively. After incubation for two hours, 400 µL termination solution was added. The samples were centrifuged at 4000 rpm for 10 min, and the supernatant was extracted. A total of 100 µL supernatant was mixed with 200 µL ultrapure water and fully shaken for 10 min. The absorption peak area (A) of the sample to be tested was determined by HPLC.
4.5. Pharmacokinetic Experiment of N6

Six male SD rats were randomly divided into two groups (administered with N6 and NVR 3-778, respectively). The rats were fasted for 12 h before administration and had free access to water. Animal feed was given 4 h after administration. Compounds N6 and NVR 3-778 were both administered orally at a dose of 10 mg/kg. The corresponding doses of compounds were dissolved in DMSO/PEG400/water (3%/60%/37%, V/V/V) mixed solution to prepare compound oral solutions, respectively, which were filtered through the 0.2 µM filter. After intragastric administration, blood was obtained by jugular vein puncture (blood point: before administration and 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 10 h, and 24 h after administration). Then 0.25 mL whole blood was collected from each rat at each time point, adding EDTA-K2 for anticoagulation. After collection, the plasma was centrifuged within 1 h (centrifugal condition: 8000 rpm, 5 min, 8 °C). Plasma samples were stored in a refrigerator before analysis. After the experiment, all rats were executed according to the regulations of the Experimental Animal Ethics Committee.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27185987/s1, Figures: Mass spectrum, hydrogen spectrum, and carbon spectrum of all end products.

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