ORIGINAL RESEARCH

Synthesis of 4-oxotetrahydropyrimidine-1(2H)-carboxamides derivatives as capsid assembly modulators of hepatitis B virus

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Abstract

We report herein the synthesis and evaluation of phenyl ureas derived from 4-oxotetrahydropyrimidine as novel capsid assembly modulators of hepatitis B virus (HBV). Among the derivatives, compound **27** (**58031**) and several analogs showed an activity of submicromolar EC_{50} against HBV and low cytotoxicities (>50 μM). Structure–activity relationship studies revealed a tolerance for an additional group at position 5 of 4-oxotetrahydropyrimidine. The mechanism study indicates that compound **27** (**58031**) is a type II core protein allosteric modulator (CpAMs), which induces core protein dimers to assemble empty capsids with fast electrophoresis mobility in native agarose gel. These compounds may thus serve as leads for future developments of novel antivirals against HBV.

Keywords 4-Oxotetrahydropyrimidine · Phenyl ureas · Hepatitis B virus · Capsid assembly

Introduction

Hepatitis B virus (HBV) chronically infects 258 million people worldwide and causes 880 thousand deaths annually due to cirrhosis, hepatocellular carcinoma, and liver failure [1]. The current standard of care medications, including pegylated interferon alpha that regulates host antiviral immune response and nucleos(t)ide analogues (NUCs) that inhibit viral DNA polymerase, can potently suppress viral

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replication, but fail to induce the loss of HBV surface antigen (HBsAg), an indication of successful immune control or the functional cure of chronic hepatitis B, in the vast majority of the treated patients [2, 3]. Therefore, development of novel antivirals targeting other steps of HBV replication as well as drugs that can activate host antiviral immune response is required to achieve the functional cure of chronic hepatitis B [4, 5]. Particularly, selective packaging of viral pregenomic (pg) RNA–DNA polymerase complex by 120 core protein (Cp) dimers into a nucelocapsid for viral DNA synthesis to take place is a key step of HBV replication and thus an ideal target for novel antiviral development [6]. In the last two decades, multiple small molecule inhibitors of HBV pgRNA encapsidation have been discovered and several leads from three chemotypes—heteroaryldihydropyrimidine (HAPs, **1**), dibenzothiazepine derivatives (DBTs, **2**), and sulfamoylbenzamides (SBAs, **3**)—have been extensively developed and are currently in clinical trials for the treatment of chronic hepatitis B (Figs 1 and 2) [6, 7]. Mechanistically, all the structurally diversified capsid assembly modulators or core protein allosteric modulators (CpAMs) bind to a hydrophobic pocket (HAP pocket) between Cp dimer–dimer interfaces to misdirect the assembly of Cp dimers into non-capsid polymers (type I CpAM) or morphologically "normal" capsids devoid of pgRNA and viral DNA polymerase (type II CpAM) [6, 8]. Of over a dozen families of CpAMs discovered thus far,
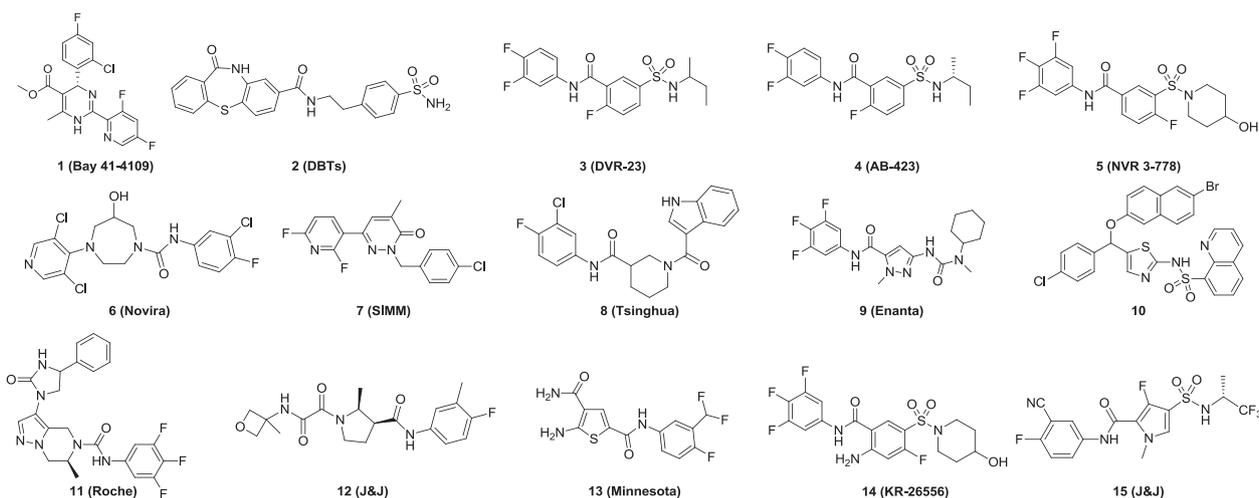
Fig. 1 Representative capsid modulators from the three major chemotype families, and modulators with the feature of two side chains meta to each other, which is similar to SBAs
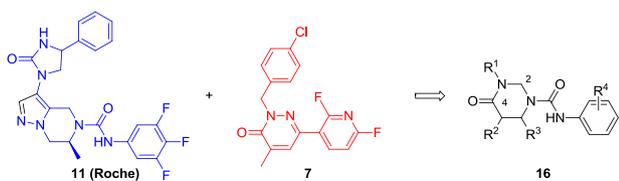

Fig. 2 Rationale of the design of 4-oxo-tetrahydropyrimidine-derived phenyl ureas

SBAs have received much attention due to their structural simplicity, availability of the cocrystal structures with capsids or Y132A mutant Cp heximers, and potential for structural modifications [9, 10].

Extensive SARs have been reported for SBAs [7]. Early works on SBAs focused on the optimization of the two side chains out of the central phenyl ring, sulfonamide and benzamide, that are meta to each other, and two clinical candidates were produced (**4** and **5**, Fig. 1) [11–14]. Further investigation has turned the attention to modifying the central core, because the angles and trajectories of the two side chains partially depend on how they are connected to and the shape of the central cores, and the trajectory and conformation of the side chains are important for directing their interactions with the target amino acid units, even at the same HAP pocket between Cp dimer–dimer interfaces. Several new central cores ranging from 7-membered to 5-membered rings, saturated and unsaturated, have been reported including aniline and pyrrole, which has led to more potent lead compounds, such as **14** and **15** (Fig. 1) [15–24]. Although they are promising, considering different scaffolds may confer different resistance profiles due to the interaction with different amino acid residues of Cp at HAP pocket [10, 25], so the identification of new central cores that can serve as bases for directing new

interactions remains necessary. Here we report the design, synthesis, and characterization of phenyl ureas based on a new central core, 4-oxo-tetrahydropyrimidine, as nevol CpAMs.

4-Oxo-tetrahydropyrimidine is a pharmacophore that can be directly derivatized to put forward two branch side chains from the two nitrogen atoms separated by a methylene. The introduction of other groups at the 5-,6-carbons is also achievable. This moiety has been used in the preparation of other biologically active compounds, such as p38 MAP kinase inhibitors [26], cell adhesion inhibitors [27], metalloprotease inhibitors [28], MMP2, MMP3, and MMP9 inhibitors [29], and aspartyl protease inhibitors [30]. We envisioned that we could introduce an aryl group at N1 through a urea linker to mimic the benzamide side chain in some of the capsid modulators and another group at N3 to explore additional bindings with the target. This structure can also be viewed as a combination of the partial pyrazine compound **11** with partial pyridazinone **7**.

Results and discussion

Synthesis

A general synthetic route for the compounds is illustrated in Scheme 1. A Boc-protected β-amino acid was coupled with an amine to form an amide **18**. Next, the Boc was removed with HCl to afford intermediate **19**, followed by cyclization with paraformaldehyde in the presence of NaOH or cyanuric chloride to generate the 4-oxo-tetrahydropyrimidine core **20** [31, 32], which could be reacted with phenyl carbamates to afford the desired phenyl ureas for evaluation.
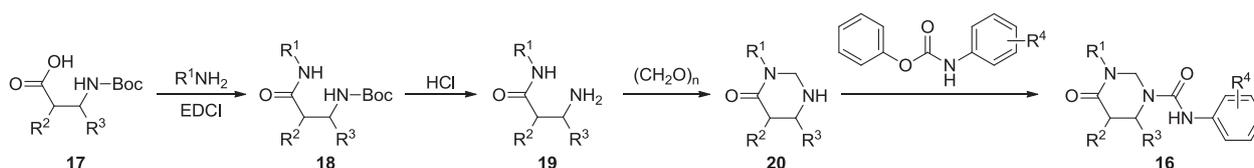
Scheme 1 General route for the synthesis of phenyl ureas derived from 4-oxotetrahydropyrimidine

Biological evaluation of new compounds. The antiviral activity of compounds was tested in an immortalized mouse hepatocyte (AML12)-derived stable cell line (AML12HBV10) that supports a high level of HBV replication. The effect of compounds on HBV-DNA replication in this cell line was determined by a dot-blot hybridization assay, which is the platform for our initial high throughput screening of HBV replication inhibitors, which resulted in the discovery of three chemotypes of CpAMs. Taking the advantage of its high throughput property, AML12HBV10-based antiviral and cytotoxicity assays were used to determine EC_{50} and CC_{50} of new compounds and direct the structure–activity relationship (SAR) study [9]. The antiviral activity of selected compounds was further confirmed in a human hepatoma-derived stable cell line supporting HBV replication (HepDES19). The mode of action of representative compounds on capsid assembly and pgRNA encapsidation was also investigated in hepatocytes by examination of capsid electrophoresis mobility and capsid-associated viral DNA as well as Cp dephosphorylation, a process-associated with pgRNA encapsidation.

In our previous work on benzamide CpAMs, we identified 3-chloro-4-fluoroaniline as a suitable fragment for the amine part of the amide [33]. We applied this aniline at the 1-position of the 4-oxo-tetrahydropyrimidine via a urea linker, and explored the effect of R^1 substitution at the 3-position first (Table 1). A para-fluorobenzene connected to the benzene through one methyl is slightly more potent than one connected through a two-methylene linker (EC_{50}, 2.02 μM of 21 vs. EC_{50}, 5.08 μM of 22). Fluorine scan and walking based on 21 provided compound 27 (58031), with a fourfold potency increase (EC_{50}, 0.52 μM) and no cytotoxicity at 50 μM. Interestingly, this 2,4-disubstitution pattern worked well when dimethoxy was evaluated in 28, albeit with a slightly weaker potency observed compared to 27 (58031). The basic and more hydrophilic pyridine was also evaluated at that position, but rendered compounds 29 and 30 less active. An attempt to extend the side chain through five-membered rings as used in the optimization of compound 11, resulting in compound 32 and 33, failed to improve the potencies. The (2,4-difluorophenyl)methylene group at position 3 in 27 (58031) was thus considered a proper starting point for SAR at other positions.

3-Chloro-4-fluoroaniline was found optimal in our previous benzamide optimization [33], but more anilines have been used and have shown unique physiochemical properties since then [22]. The variation of R4 was therefore investigated (Table 2). 3-chloroaniline in 35 and 3-(difluoromethyl)-4-fluoroaniline in 36 were found to have comparable activities to 27 (58031), while the tri-halogen substitution in 40, 41, and 42 reduced the activities. The replacement of the aniline with an ortho-fluorobenzylamine in 43 was not tolerated.

The effect of adding functional groups at the 5- and 6-positions of the central 4-oxo-tetrahydropyrimidine core was also explored. The assessment of substitutions at the 5- and 6- positions was performed based on 27 (58031) (Table 3). Among the small number of groups tested at the 6-position (R^3), the methyl group resulted in reduced activity, suggesting that compound 44 does not have the same SAR as compound 7, in which a methyl substitution next to the endocyclic carbonyl group has a beneficial impact to the activity [16]. The introduction of a benzyl group, or 4,4-difluoropiperidine or 3,3-difluoropyrrolidine through a methylene linker, deteriorated the potency of compounds 45, 46, and 47. In contrast, substitutions at the 5-position were shown to be more tolerable. The compounds with substituents like phenyl in 50, triazole in 52, benzamide in 53, sulfonamide in 54, and acrylamide in 55 displayed comparable or slightly better potencies than 27 (58031), while the introduction of a dimethyl group, a fused cyclopentane, and a benzyl group diminished the activities only by two to threefold, as observed in 48, 49, and 51. The compounds 52, 53, 54, and 55 were prepared from 2-azido-3-((tert-butoxycarbonyl)amino)propanoic acid 56 according to the Scheme 1 to form the azide intermediate, 5-azido-N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide 60. This azide 60 underwent cyclization with ethynylcyclopropane to form 52, or was reduced to 5-amino-N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide 61, from which benzamide in 53 and sulfonamide in 54 were introduced (Scheme 2). The acceptance for the addition of functional groups at the 5-position (R^2) indicated that this family of compounds does not have the same SAR as the structurally similar compounds 7 and 11, and that it can be further explored for better leads.

The anti-HBV activity of 27 (58031) was further assessed in a human hepatoma-derived cell line

Table 1 SAR study of substituents at 3-nitrogen

Cmpd	21	22	23	24	25	26	27 (58031)
R^1	(structure)	(structure)	(structure)	(structure)	(structure)	(structure)	(structure)
EC$_{50}$[a] (μM)	2.02	5.08	4.50	> 10	7.79	1.50	0.52
CC$_{50}$[b] (μM)	> 50	> 50	> 50	> 50	> 50	> 50	> 50
Cmpd	28	29	30	31	32	33	
R^1	(structure)	(structure)	(structure)	(structure)	(structure)	(structure)	
EC$_{50}$ (μM)	0.97	> 10	6.59	1.50	2.76	> 10	
CC$_{50}$ (μM)	> 50	> 50	> 50	> 50	> 50	> 50	

38017 [33] was used as a positive control

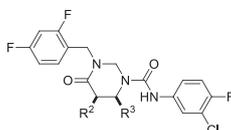[a]EC50 is 50% inhibitory concentration of cytoplasmic HBV-DNA replication

[b]CC50 is 50% cytotoxicity concentration in AML 12HBV10 cells; all tests were run in duplicates
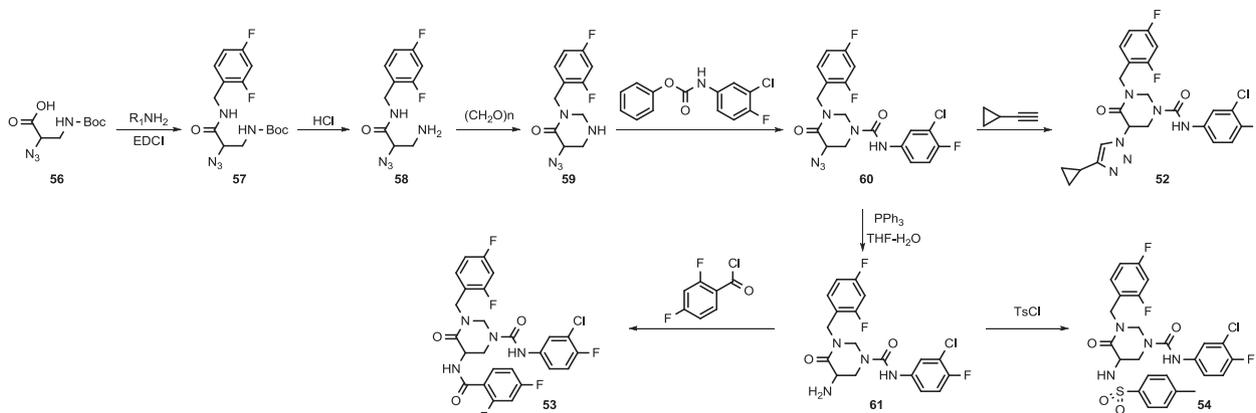
Table 2 SAR study of anilines

Cmpd	34	35	36	37	38
R^4	(structure)	(structure)	(structure)	(structure)	(structure)
EC$_{50}$ (μM)	1.88	0.47	0.50	4.92	2.88
CC$_{50}$ (μM)	> 50	> 50	> 50	> 50	> 50
Cmpd	39	40	41	42	43
R^4	(structure)	(structure)	(structure)	(structure)	(structure)
EC$_{50}$ (μM)	> 10	3.10	1.36	> 10	> 10
CC$_{50}$ (μM)	> 50	> 50	> 50	> 50	> 50

HepDES19 and demonstrated to inhibit HBV-DNA replication in a concentration-dependent manner with EC$_{50}$ value of 0.84 (Fig. 3), which is slightly higher that its EC$_{50}$ value in AML12HBV10 cells. Moreover, similar to ENAN-342017, a SBA chemotype of type II CpAM [34], treatment of AML12HBV_DE11 cells with **27** (**58031**) induced the assembly capsids with faster electrophoresis mobility in a native agarose gel and drastically reduced the amount of capsid-associated viral DNA (Fig. 4A) [35]. As anticipated, **Bay 41-4109**, a type I CpAM, inhibited capsid assembly and subsequent HBV-DNA synthesis. Also as expected, treatment of AML12HBV_DE11 cells with HBV-DNA polymerase inhibitor entecavir (ETV) did not alter capsid

Table 3 SAR study of substitutions at 5- and 6- position

Cmpd	44	45	46	47	48	49
R^2	H	H	H	H	Me, Me	
R^3	Me				H	
EC$_{50}$ (μM)	4.82	2.39	9.62	> 10	1.08	1.82
CC$_{50}$ (μM)	> 50	> 50	> 50	> 50	> 50	> 50
Cmpd	50	51	52	53	54	55
R^2						
R^3	H	H	H	H	H	H
EC$_{50}$ (μM)	0.46	1.66	0.54	0.38	0.38	0.43
CC$_{50}$ (μM)	> 50	> 50	> 50	> 50	> 50	> 50

Scheme 2 Synthesis of analogs of **27** derived from 5-amine

electrophoresis mobility, but significantly inhibited the synthesis of HBV DNA. These results indicate that **27** (**58031**) is a typical type II CpAM that misdirects the assembly of Cp dimers into empty capsids and thus precludes the synthesis of viral DNA. In support of this notion, similar to **ENAN-34017** or **Bay 41-4109**, **27** (**58031**) treatment inhibited Cp dephosphorylation (Fig. 4B), which is catalyzed by cellular protein phosphatase 1 during pgRNA encapsidation and essential for the assembly of pgRNA-containing nucelocapsids, but not empty capsids. As anticipated, ETV treatment did not affect Cp dephosphorylation [36, 37].

Conclusions

In summary, the authors designed and synthesized 4-oxotetrahydropyrimidine-derived phenyl ureas as a new chemotype of CpAMs. SAR studies at four positions of the central core resulted in the discovery of compound **27** (**58031**) and several other analogs with submicromolar activities. Compound **27** (**58031**) was found to inhibit HBV in mouse and human hepatocytes and its mode of action is consistent with typical type II CpAM, i.e., misdirect the Cp dimers to assembly empty capsids devoid of pgRNA and thus precludes the synthesis of viral DNA.
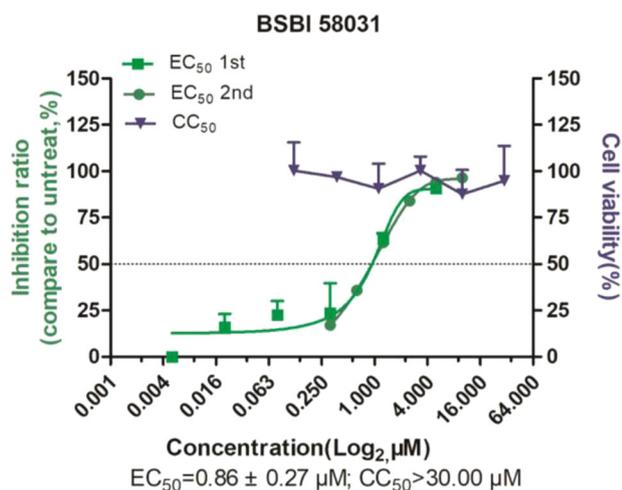
Fig. 3 Compound **58031** inhibits HBV replication in HepDES19 cells. The cells were treated with a serial twofold dilution of **58031** for 4 days. Intracellular HBV DNA were quantified by a qPCR assay. EC_{50} was calculated from two independent biologically triplicate experiments. Cell viability was determined by MTT assay and CC_{50} was calculated from a biologically triplicate experiment

Fig. 4 Compound **58031** is a type II CpAM. AML12HBV_DE11 cells were cultured in the presence of tet (tet+) or cultured in the absence of tet and mock treated (tet-) or treated with the indicated concentrations of **27** (**58031**), 2 μM of **ENAN-34017**, 2 μM of **Bay 41-4109** or 1 μM of entecavir (ETV) for 30 h. **A** HBV capsids and capsid-associated viral DNA were detected. Slow and fast migrating capsids were indicated. **B** Hyper-phopshorylated and dephosphorylated (or hypo-phosphorylated) HBV core protein were detected by a western blot assay, with β-actin as a loading control

Materials and methods

Chemistry

All reagents and solvents were used as purchased from commercial sources. Reactions were carried out under argon atmosphere. Flash column chromatography was performed on either CombiFlash Rf+ or CombiFlash Companion using the appropriate size Teledyne ISCO columns (20–40 μm or 40–60 μm) and prepacked silica filled cartridges. Preparative high-performance liquid chromatography (HPLC) was performed using a Gilson 331 and 332 pumps with a UV/VIS-155 detector and GX-271 liquid handler. Column was Phenomenex Luna LC Column (5 μm C18 100 Å, 150 × 21.2 mm). ^{1}H NMR spectra were recorded on a 300 MHz INOVA VARIAN spectrometer. Chemical shifts values are given in ppm and referred against the internal standard of TMS (tetramethylsilane). The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; q, quadruplet; m, multiplet and dd, doublet of doublets. The coupling constants (J) are reported in Hertz (Hz). Mass Spectra were obtained on an Agilent 6120 mass spectrometer with electrospray ionization source (1200 Aligent LC-MS spectrometer, Positive). Mobile phase flow was 1.0 mL/min with a 3.0 min gradient from 20% aqueous media (0.1% formic acid) to 95% CH_3CN (0.1% formic acid) and a 9.0 min total acquisition time. All the tested compounds possess a purity of at least 95%, which was determined by LC/MS Data recorded using an Agilent 1200 liquid chromatography and Agilent 6120 mass spectrometer, and further supported by clean NMR spectra.
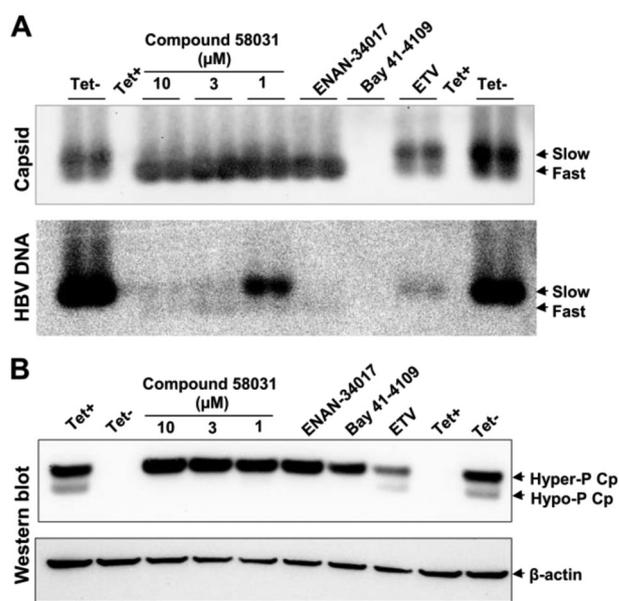
N-(3-chloro-4-fluorophenyl)-3-(4-fluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (21)

4-fluorobenzylamine (0.264 mmol), EDC·HCl (51 mg, 0.264 mmol), HOBt·H$_2$O (40 mg, 0.264 mmol), and excess triethylamine was added to Boc-beta-Ala-OH (50 mg, 0.264 mmol) in 2 mL DCM. The reaction was stirred overnight. After diluting with EtOAc, the reaction mixture was washed with saturated aqueous $NaHCO_3$ and then brine. The organic phase was dissolved in 1:1 MeOH to 4 M HCl in dioxane for several hours and then concentrated down. After drying on high vacuum overnight, the intermediate was refluxed in EtOH at 70 °C overnight with paraformaldehyde (10 mg, 0.343 mmol) and 10 N aqueous NaOH (42 μL, 0.422 mmol). The finished reaction was diluted with EtOAc and washed with brine twice. The organic phase was redissolved in DCM with excess Et$_3$N to react with phenyl (3-chloro-4-fluorophenyl)carbamate, which was synthesized by reacting 3-chloro-4-fluoroaniline with 1.2 eq of phenyl chloroformate in 1:1 EtOAc to saturate aqueous $NaHCO_3$ overnight and then purifying the concentrated organic phase with CombiFlash or HPLC. The reaction was diluted with EtOAc and washed with 2 M HCl thrice, saturated aqueous $NaHCO_3$ once, and brine once. The desired product **21** (15.7 mg, 16%) was obtained after HPLC separation. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.49–7.40 (m, 1H),

7.29–7.25 (m, 1H), 7.24–7.19 (m, 1H), 7.19–7.10 (m, 1H), 7.06–6.93 (m, 3H), 4.82–4.76 (m, 2H), 4.60–4.54 (m, 2H), 3.73–3.63 (m, 2H), 2.70–2.60 (m, 2H); Calculated for $C_{18}H_{16}ClF_2N_3O_2$, 379.1; observed $(M + H)^+$ 380.5.

N-(3-chloro-4-fluorophenyl)-3-(4-fluorophenethyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (22)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (50 mg, 0.264 mmol) was treated with 2-(4-fluorophenyl)ethanamine (0.264 mmol), EDC·HCl (51 mg, 0.264 mmol), triethylamine, and HOBt·H$_2$O (40 mg, 0.264 mmol), and then continued the same procedure to afford **22** (3.2 mg, 3%). ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.53–7.46 (m, 1H), 7.23–7.16 (m, 1H), 7.16–7.08 (m, 2H), 7.08–6.98 (m, 1H), 6.98–6.87 (m, 1H), 4.71 (s, 2H), 3.64–3.50 (m, 4H), 2.83 (t, $J = 7.2$ Hz, 2H), 2.54–2.49 (m, 2H); Calculated for $C_{19}H_{18}ClF_2N_3O_2$, 393.1; observed $(M + H)^+$ 394.5.

N-(3-chloro-4-fluorophenyl)-3-(3-fluorophenethyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (23)

According to the procedure for preparation of compound 21, Boc-beta-Ala-OH (50 mg, 0.264 mmol) was treated with 2-(3-fluorophenyl)ethanamine (0.264 mmol), EDC·HCl (51 mg, 0.264 mmol), triethylamine, and HOBt·H$_2$O (40 mg, 0.264 mmol), and then continued the same procedure to afford **23** (3.7 mg, 4%). ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.52–7.44 (m, 1H), 7.24–7.14 (m, 2H), 7.06–7.00 (m, 1H), 7.00–6.91 (m, 1H), 6.91–6.78 (m, 2H), 4.69 (s, 2H), 3.65–3.50 (m, 4H), 2.90–2.83 (m, 2H), 2.58–2.48 (m, 2H); Calculated for $C_{19}H_{18}ClF_2N_3O_2$, 393.1; observed $(M + H)^+$ 394.5.

N-(3-chloro-4-fluorophenyl)-3-(2-morpholinoethyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (24)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (100 mg, 0.529 mmol) was treated with 2-morpholinoethanamine (76 μL, 0.581), DCC (120 mg, 0.581 mmol), and HOBt·H$_2$O (89 mg, 0.581 mmol), and then continued the same procedure to afford **24** (1.7 mg, 2%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.72–7.66 (m, 1H), 7.28–7.20 (m, 1H), 7.20–7.10 (m, 1H), 4.56 (s, 2H), 4.13–3.95 (m, 2H), 3.95–3.70 (m, 4H), 3.70–3.35 (m, 4H), 3.28–3.04 (m, 4H), 2.68–2.58 (m, 1H), 2.45–2.35 (m, 1H); Calculated for $C_{12}H_{22}ClFN_4O_3$, 384.1; observed $(M + H)^+$ 385.5.

N-(3-chloro-4-fluorophenyl)-3-(3,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (25)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (50 mg, 0.264 mmol) was treated with

3,4-difluorobenzylamine (0.264 mmol), EDC·HCl (51 mg, 0.264 mmol), triethylamine, and HOBt·H$_2$O (40 mg, 0.264 mmol), and then continued the same procedure to afford **25** (8.0 mg, 8%). ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.49–7.44 (m, 1H), 7.21–7.13 (m, 2H), 7.13–7.00 (m, 3H), 4.83 (s, 2H), 4.56 (s, 2H), 3.70 (t, $J = 6.6$ Hz, 2H), 2.68 (t, $J = 6.6$ Hz, 2H); Calculated for $C_{18}H_{15}ClF_3N_3O_2$, 397.1; observed $(M + H)^+$ 398.5.

N-(3-chloro-4-fluorophenyl)-3-(2,5-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (26)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (50 mg, 0.264 mmol) was treated with 2,5-difluorobenzylamine (0.264 mmol), EDC·HCl (51 mg, 0.264 mmol), triethylamine, and HOBt·H$_2$O (40 mg, 0.264 mmol), and then continued the same procedure to afford **26** (13.7 mg, 13%). ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.48–7.40 (m, 1H), 7.21–7.12 (m, 1H), 7.04–6.84 (m, 4H), 4.85 (s, 2H), 2.59 (s, 2H), 3.72–3.60 (m, 2H), 2.65–2.54 (m, 2H); Calculated for $C_{18}H_{15}ClF_3N_3O_2$, 397.1; observed $(M + H)^+$ 398.5.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (27, 58031)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (200 mg, 1.057 mmol) was treated with 2,4-difluorobenzylamine (0.14 mL, 1.163 mmol), DCC (240 mg, 1.163 mmol), and HOBt·H$_2$O (178 mg, 1.163 mmol), and then continued the same procedure to afford **27** (**58031**) (159.4 mg, 38%). ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.49–7.43 (m, 1H), 7.41–7.31 (m, 1H), 7.18–7.11 (m, 1H), 7.10–7.01 (m, 1H), 6.89–6.76 (m, 2H), 6.50 (s, 1H, NH), 4.91 (s, 2H), 4.63 (s, 2H), 3.70 (t, $J = 6.6$ Hz, 2H), 2.68 (t, $J = 6.6$ Hz, 2H); 13C NMR (100 MHz) showed a conformer mixture: δ (ppm) 169.8, 162.8 (dd, $J = 249$, 12 Hz), 161.1 (dd, $J = 247$, 12 Hz), 159.0 (d, $J = 41$ Hz), 154.9 (d, $J = 245$ Hz), 154.0, 134.4 (d, $J = 3$ Hz), 132.0 (dd, $J = 10.0$, 5.4 Hz), 123.1, 121.1 (d, $J = 18$ Hz), 120.5 (d, $J = 8$ Hz), 118.7 (dd, $J = 16.4$ Hz), 116.6 (d, $J = 23$ Hz), 111.9 (dd, $J = 22$, 4 Hz), 104.1 (t, $J = 26$ Hz), 58.2, 58.1, 42.7, 42.7, 41.0, 31.3; HRMS, Calculated for $C_{18}H_{15}ClF_3N_3NaO_2$, 420.0697; observed $(M + Na)^+$ 420.0687.

N-(3-chloro-4-fluorophenyl)-3-(2,4-dimethoxybenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (28)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (100 mg, 0.529 mmol) was treated with 2,4-dimethoxybenzylamine (76 μL, 0.581 mmol), DCC (120 mg, 0.581 mmol), and HOBt·H$_2$O (89 mg, 0.581 mmol),

and then continued the same procedure to afford **28** (62.5 mg, 28%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.52–7.46 (m, 1H), 7.25–7.18 (m, 1H), 7.16–7.08 (m, 2H), 6.49–6.45 (m, 1H), 6.45–6.39 (m, 1H), 4.88 (s, 2H), 4.54 (s, 2H), 3.80 (s, 3H), 3.74–3.68 (m, 5H), 2.64 (t, $J = 6.6$ Hz, 2H); Calculated for C$_{20}$H$_{21}$ClFN$_3$O$_4$, 421.1; observed (M + H)$^+$ 422.5.

N-(3-chloro-4-fluorophenyl)-4-oxo-3-(pyridin-4-ylmethyl) tetrahydropyrimidine-1(2H)-carboxamide (29)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (100 mg, 0.529 mmol) was treated with pyridin-4-ylmethanamine (58 μL, 0.581 mmol), DCC (120 mg, 0.581 mmol), and HOBt·H$_2$O (89 mg, 0.581 mmol), and then continued the same procedure to afford **29** (5.0 mg, 3%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 8.79–8.69 (m, 2H), 7.96–7.88 (m, 2H), 7.67–7.59 (m, 1H), 7.36–7.27 (m, 1H), 7.22–7.13 (m, 1H), 5.03 (s, 2H), 4.92–4.84 (m, 2H), 3.97–3.87 (m, 2H), 2.76–2.66 (m, 2H); Calculated for C$_{17}$H$_{16}$ClFN$_4$O$_2$, 362.1; observed (M + H)$^+$ 363.4.

N-(3-chloro-4-fluorophenyl)-4-oxo-3-(pyridin-2-ylmethyl) tetrahydropyrimidine-1(2H)-carboxamide (30)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (100 mg, 0.529 mmol) was treated with pyridin-2-ylmethanamine (60 μL, 0.581 mmol), DCC (120 mg, 0.581 mmol), and HOBt·H$_2$O (89 mg, 0.581 mmol), and then continued the same procedure to afford **30** (8.9 mg, 5%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 8.70–8.66 (m, 1H), 8.35–8.26 (m, 1H), 7.86–7.80 (m, 1H), 7.78–7.71 (m, 1H), 7.64–7.59 (m, 1H), 7.36–7.28 (m, 1H), 7.21–7.12 (m, 1H), 5.07 (s, 2H), 4.89 (s, 2H), 3.90 (t, $J = 6.4$ Hz, 2H), 2.69 (t, $J = 6.4$ Hz, 2H); Calculated for C$_{17}$H$_{16}$ClFN$_4$O$_2$, 362.1; observed (M + H)$^+$ 363.5.

N-(3-chloro-4-fluorophenyl)-3-(cyclopropylmethyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (31)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (100 mg, 0.529 mmol) was treated with cyclopropylmethanamine (50 μL, 0.581 mmol), DCC (120 mg, 0.581 mmol), and HOBt·H$_2$O (89 mg, 0.581 mmol), and then continued the same procedure to afford **31** (12.4 mg, 7%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.65–7.59 (m, 1H), 7.36–7.28 (m, 1H), 7.20–7.12 (m, 1H), 5.03 (s, 2H), 4.93–4.87 (m, 2H), 3.78 (t, $J = 6.6$ Hz, 2H), 2.61 (t, $J = 6.6$ Hz, 2H), 1.10–0.97 (m, 1H), 0.58–0.49 (m, 2H), 0.33–0.25 (m, 2H); Calculated for C$_{15}$H$_{17}$ClFN$_3$O$_2$, 325.1; observed (M + H)$^+$ 326.4.

N-(3-chloro-4-fluorophenyl)-4-oxo-3-((1-phenylpyrrolidin-3-yl)methyl)tetrahydropyrimidine-1(2H)-carboxamide (32)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (60 mg, 0.317 mmol) was treated with (1-phenylpyrrolidin-3-yl)methanamine (62 mg, 0.349 mmol), DCC (72 mg, 0.349 mmol), and HOBt·H$_2$O (53 mg, 0.349 mmol), and then continued the same procedure to afford **32** (85.7 mg, 63%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.66–7.60 (m, 1H), 7.35–7.23 (m, 3H), 7.20–7.10 (m, 1H), 6.95–6.85 (m, 3H), 4.50 (s, 2H), 3.80 (t, $J = 6.5$ Hz, 2H), 3.61–3.41 (m, 6H), 2.92–2.78 (m, 1H), 2.65 (t, $J = 6.5$ Hz, 2H), 2.30–2.16 (m, 1H), 1.96–1.80 (m, 1H); Calculated for C$_{22}$H$_{24}$ClFN$_4$O$_2$, 430.2; observed (M + H)$^+$ 431.6.

N-(3-chloro-4-fluorophenyl)-4-oxo-3-((2-oxo-3-phenyloxazolidin-5-yl)methyl)tetrahydropyrimidine-1(2H)-carboxamide (33)

According to the procedure for preparation of compound **21**, Boc-beta-Ala-OH (60 mg, 0.317 mmol) was treated with 5-(aminomethyl)-3-phenyloxazolidin-2-one (80 mg, 0.349 mmol), DCC (72 mg, 0.349 mmol), and HOBt·H$_2$O (53 mg, 0.349 mmol), and then continued the same procedure to afford **33** (13.2 mg, 9%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.65–7.59 (m, 1H), 7.57–7.50 (m, 2H), 7.40–7.26 (m, 3H), 7.18–7.08 (m, 2H), 5.16–5.00 (m, 2H), 5.00–4.90 (m, 1H), 4.22–4.13 (m, 1H), 3.93–3.71 (m, 5H), 2.68–2.60 (m, 2H); Calculated for C$_{21}$H$_{20}$ClFN$_4$O$_4$, 446.1; observed (M + H)$^+$ 447.6.

3-(2,4-difluorobenzyl)-N-(4-fluoro-3-(trifluoromethyl)phenyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (34)

2,4-difluorobenzylamine (378 mg, 2.64 mmol), EDC·HCl (557 mg, 2.91 mmol), HOBt·H$_2$O (444 mg, 2.91 mmol), and excess triethylamine was added to Boc-beta-Ala-OH (500 mg, 2.64 mmol) in 10 mL DCM. The reaction was stirred overnight. After diluting with EtOAc, the reaction mixture was washed with saturated aqueous NH$_4$Cl and brine. The organic phase was dissolved in 1:1 MeOH to 4 M HCl in dioxane for several hours and then dried overnight on high vacuum. Then, it was refluxed in acetonitrile at 50 °C overnight with paraformaldehyde (1.2 eq.) and cyanuric chloride (0.1 eq.). The reaction was concentrated down and purified with HPLC to afford the cyclized intermediate, 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one. 3-(difluoromethyl)-4-fluoroaniline was reacted with 1.2 eq of phenyl chloroformate in 1:1 EtOAc to saturate aqueous NaHCO$_3$ overnight. The organic phase was concentrated down and reacted with 3-(2,4-difluorobenzyl) tetrahydropyrimidin-4(1H)-one with excess DIPEA in DCM overnight. The desired product **34** was obtained after

HPLC separation. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.61–7.54 (m, 2H), 7.39–7.28 (m, 1H), 7.15–7.06 (m, 1H), 6.86–6.74 (m, 2H), 4.88 (s, 2H), 4.61 (s, 2H), 3.70 (t, J = 6.8 Hz, 2H), 2.63 (t, J = 6.8 Hz, 2H); Calculated for C$_{19}$H$_{15}$F$_6$N$_3$O$_2$, 431.1; observed (M + H)$^+$ 432.6.

N-(3-chlorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (35)

According to the procedure for preparation of compound **34**, 3-chloroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the same procedure to afford **35**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.44–7.37 (m, 1H), 7.37–7.28 (m, 2H), 7.24–7.12 (m, 2H), 7.06–6.96 (m, 1H), 6.88–6.73 (m, 2H), 4.87 (s, 2H), 4.60 (s, 2H), 3.69 (t, J = 6.6 Hz, 2H), 2.63 (t, J = 6.6 Hz, 2H); Calculated for C$_{18}$H$_{16}$ClF$_2$N$_3$O$_2$, 379.1; observed (M + H)$^+$ 380.6.

3-(2,4-difluorobenzyl)-N-(3-(difluoromethyl)-4-fluorophenyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (36)

According to the procedure for preparation of compound **34**, 3-(difluoromethyl)-4-fluoroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the same procedure to afford **36**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.55–7.45 (m, 2H), 7.36–7.25 (m, 1H), 7.07–6.98 (m, 1H), 6.86–6.73 (m, 2H), 7.02–6.60 (m, 1H, CHF$_2$, broad), 4.87 (s, 2H), 4.60 (s, 2H), 3.69 (t, J = 6.6 Hz, 2H), 2.62 (t, J = 6.6 Hz, 2H); Calculated for C$_{19}$H$_{16}$F$_5$N$_3$O$_2$, 413.1; observed (M + H)$^+$ 414.6.

3-(2,4-difluorobenzyl)-N-(2,3-dimethylphenyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (37)

According to the procedure for preparation of compound **34**, 3-(difluoromethyl)-4-fluoroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the procedure of compound **27** to afford **37**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.36–7.28 (m, 1H), 7.12–6.94 (m, 3H), 6.85–6.70 (m, 2H), 4.85 (s, 2H), 4.59 (s, 2H), 3.68 (t, J = 6.6 Hz, 2H), 2.74 (t, J = 6.6 Hz, 2H), 2.25 (s, 3H), 2.04 (s, 3H); Calculated for C$_{20}$H$_{21}$F$_2$N$_3$O$_2$, 373.2; observed (M + H)$^+$ 374.6.

3-(2,4-difluorobenzyl)-N-(3,4-difluorophenyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (38)

According to the procedure for preparation of compound **34**, 3,4-difluoroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the procedure of compound **27** to afford **38**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.57–7.45 (m, 1H), 7.37–7.25 (m, 2H), 7.20–7.10 (m, 2H), 7.06–6.93 (m, 1H), 4.82 (s, 2H), 4.51 (s, 2H), 3.75–3.65 (m, 2H), 2.55–2.30 (m, 2H); Calculated for C$_{18}$H$_{15}$F$_4$N$_3$O$_2$, 381.1; observed (M + H)$^+$ 382.5.

N-(2,4-dichlorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (39)

According to the procedure for preparation of compound **34**, 2,4-dichloroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the procedure of compound **27** to afford **39**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.90–7.84 (m, 1H), 7.34–7.29 (m, 1H), 7.20–7.14 (m, 2H), 6.85–6.69 (m, 3H), 4.87 (s, 2H), 4.58 (s, 2H), 3.70 (t, J = 6.6 Hz, 2H), 2.66 (t, J = 6.6 Hz, 2H); Calculated for C$_{18}$H$_{15}$Cl$_2$F$_2$N$_3$O$_2$, 413.0; observed (M + H)$^+$ 414.5.

3-(2,4-difluorobenzyl)-4-oxo-N-(3,4,5-trifluorophenyl)tetrahydropyrimidine-1(2H)-carboxamide (40)

According to the procedure for preparation of compound **34**, 3,4,5-trifluoroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the procedure of compound **27** to afford **40**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.40–7.20 (m, 1H), 7.15–7.03 (m, 2H), 6.86–6.72 (m, 2H), 4.84 (s, 2H), 4.59 (s, 2H), 3.71–3.61 (m, 2H), 2.65–2.55 (m, 2H); Calculated for C$_{18}$H$_{14}$F$_5$N$_3$O$_2$, 399.1; observed (M + H)$^+$ 400.5.

N-(2-chloro-4,5-difluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (41)

According to the procedure for preparation of compound **34**, 2-chloro-4,5-difluoroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the procedure of compound **27** to afford **41**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.89–7.78 (m, 1H), 7.34–7.20 (m, 1H), 6.95–6.84 (m, 1H), 6.84–6.70 (m, 2H), 4.85 (s, 2H), 4.57 (s, 2H), 3.72–3.60 (m, 2H), 2.66–2.57 (m, 2H); Calculated for C$_{18}$H$_{14}$ClF$_4$N$_3$O$_2$, 415.1; observed (M + H)$^+$ 416.5.

N-(3,5-dichloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (42)

According to the procedure for preparation of compound **34**, 3,5-dichloro-4-fluoroaniline was treated with phenyl

chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the procedure of compound **27** to afford **42**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.41–7.33 (m, 2H), 7.33–7.26 (m, 1H), 6.86–6.72 (m, 2H), 4.88–4.80 (m, 2H), 4.59 (s, 2H), 3.70–3.60 (m, 2H), 2.64–2.54 (m, 2H); Calculated for C$_{18}$H$_{14}$Cl$_2$F$_3$N$_3$O$_2$, 431.0; observed (M + H)$^+$ 432.5.

3-(2,4-difluorobenzyl)-N-(2-fluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (43)

According to the procedure for preparation of compound **34**, 2-fluoroaniline was treated with phenyl chloroformate, and then reacted with the intermediate 3-(2,4-difluorobenzyl)tetrahydropyrimidin-4(1H)-one from the procedure of compound **27** to afford **43**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.31–7.27 (m, 2H), 7.24–7.17 (m, 1H), 7.10–6.94 (m, 2H), 6.82–6.69 (m, 2H), 4.80 (s, 2H), 4.56 (s, 2H), 4.39 (s, 2H), 3.53 (t, J = 6.6 HZ, 2H), 2.55 (t, J = 6.6 Hz, 2H); Calculated for C$_{19}$H$_{18}$F$_3$N$_3$O$_2$, 377.1; observed (M + H)$^+$ 378.5.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-6-methyl-4-oxotetrahydropyrimidine-1(2H)-carboxamide (44)

According to the procedure for preparation of compound **21**, Boc-beta-HoAla-OH (50 mg, 0.246 mmol) was treated with 2,4-difluorobenzylamine (32 µL, 0.271 mmol), DCC (56 mg, 0.271 mmol), and HOBt·H$_2$O (41 mg, 0.271 mmol), and then continued the same procedure to afford **44** (23.3 mg, 23%). ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.46–7.40 (m, 1H), 7.35–7.27 (m, 1H), 7.20–7.10 (m, 1H), 7.06–6.96 (m, 1H), 6.86–6.70 (m, 2H), 5.20–5.12 (m, 1H), 4.74–4.64 (m, 1H), 4.56–4.46 (m, 2H), 4.26–4.14 (m, 1H), 2.80–2.70 (m, 1H), 2.40–2.30 (m, 1H), 1.30–1.24 (m, 3H); Calculated for C$_{19}$H$_{17}$ClF$_3$N$_3$O$_2$, 411.1; observed (M + H)$^+$ 412.4.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxo-6-(2,4,5-trifluorobenzyl)tetrahydropyrimidine-1(2H)-carboxamide (45)

According to the procedure for preparation of compound **21**, (R)-3-((tert-butoxycarbonyl)amino)-4-(2,4,5-trifluorophenyl)butanoic acid (60 mg, 0.180 mmol) was treated with 2,4-difluorobenzylamine (24 µL, 0.198 mmol), DCC (41 mg, 0.198 mmol), and HOBt·H$_2$O (30 mg, 0.198 mmol), and then continued the same procedure to afford **45**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.44–7.8 (m, 2H), 7.12–6.90 (m, 4H), 6.90–6.72 (m, 2H), 5.14–5.06 (m, 1H), 4.76–4.64 (m, 1H), 4.64–4.52 (m, 2H), 4.34–4.22 (m, 1H), 3.09–2.98 (m, 1H), 2.78–2.65 (m, 2H), 2.50–2.38 (m, 1H); Calculated for C$_{25}$H$_{18}$ClF$_6$N$_3$O$_2$, 541.1; observed (M + H)$^+$ 542.6.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-6-((4,4-difluoropiperidin-1-yl)methyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (46)

2,4-difluorobenzylamine (0.12 mL, 1.003 mmol), DCC (207 mg, 1.003 mmol), and HOBt·H$_2$O (154 mg, 1.003 mmol) was added to 3-((tert-butoxycarbonyl)amino)-4-hydroxybutanoic acid (200 mg, 0.912 mmol) in 5 mL DCM, and stirred overnight. After filtering, the filtrate was purified by CombiFlash to afford tert-butyl (4-((2,4-difluorobenzyl)amino)-1-hydroxy-4-oxobutan-2-yl)carbamate. Oxalyl chloride (0.16 mL, 1.824 mmol) and DMSO (0.19 mL, 2.73 mmol) was dissolved in 8 mL DCM at −78 °C in a dry ice/acetone bath. After 20 min, tert-butyl (4-((2,4-difluorobenzyl)amino)-1-hydroxy-4-oxobutan-2-yl)carbamate in 7 mL DCM was added dropwise. After 2 h, triethylamine (0.51 mL, 3.65 mmol) was added dropwise, and the reaction was stirred overnight, allowing it to gradually go to room temperature. The completed reaction was quenched with saturated aqueous NH$_4$Cl, and then extracted with DCM twice to afford tert-butyl (4-((2,4-difluorobenzyl)amino)-1,4-dioxobutan-2-yl)carbamate. 4,4-difluoropiperidine hydrochloride (131 mg, 0.912 mmol) in 2 mL DCE was stirred with a few drops of triethylamine to release it from the HCl salt, and then NaBH(OAc)$_3$ (290 mg, 1.368 mmol) was added. After stirring for several minutes, tert-butyl (4-((2,4-difluorobenzyl)amino)-1,4-dioxobutan-2-yl)carbamate in 2 mL DCE was added dropwise. After letting the reaction stir overnight, it was quenched with saturated aqueous NaHCO$_3$ and stirred vigorously. The phases were separated, and the aqueous phase was extracted with DCM twice. All the organic phases were combined and purified by CombiFlash. It was then dissolved in 1:1 MeOH (2 mL) to 4 M HCl in dioxane (2 mL) for 1 h, and then dried on high vacuum. The residue was refluxed in 4 mL EtOH at 70 °C overnight with 10 N aqueous NaOH (73 µL, 0.730 mmol) and paraformaldehyde (18 mg, 0.593 mmol). The reaction was diluted with EtOAc and washed with brine. The organic phase was concentrated down and dissolved in acetonitrile. DIPEA and 4-DMAP was added, along with phenyl (3-chloro-4-fluorophenyl)carbamate. After refluxing at 110 °C overnight, the reaction was diluted with EtOAc and washed with 2 N HCl thrice, saturated NaHCO$_3$ once, and brine once. It was purified by CombiFlash to afford **46**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.43–7.38 (m, 1H) 7.37–7.32 (m, 1H), 7.08–6.98 (m, 2H), 6.86–6.70 (m, 2H), 5.24–5.16 (m, 1H), 4.90–4.78 (m, 1H), 4.50–4.34 (m, 2H), 4.32–4.15 (m, 1H), 3.05–2.74 (m, 6H), 2.72–2.58 (m, 2H), 2.40–2.28 (m, 1H), 2.16–1.96 (m, 4H); Calculated for C$_{24}$H$_{24}$ClF$_5$N$_4$O$_2$, 530.2; observed (M + H)$^+$ 531.6.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-6-((3,3-difluoropyrrolidin-1-yl)methyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (47)

According to the procedure for preparation of compound **46**, 3,3-difluoropyrrolidine hydrochloride was used instead of 4,4-difluoropiperidine hydrochloride to afford **47** (8.5 mg, 2%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.50–7.44 (m, 1H), 7.42–7.30 (m, 1H), 7.18–7.10 (m, 2H), 6.93–6.82 (m, 2H), 5.18–5.12 (m, 1H), 4.84–4.68 (m, 3H), 4.62–4.53 (m, 1H), 3.30–2.90 (m, 6H), 2.84–2.74 (m, 1H), 2.66–2.37 (m, 4H); Calculated for C$_{23}$H$_{22}$ClF$_5$N$_4$O$_2$, 516.1; observed (M + H)$^+$ 517.6.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-5,5-dimethyl-4-oxotetrahydropyrimidine-1(2H)-carboxamide (48)

According to the procedure for preparation of compound **21**, 3-((tert-butoxycarbonyl)amino)-2,2-dimethylpropanoic acid (37 mg, 0.168 mmol) was treated with 2,4-difluorobenzylamine (20 μL, 0.168 mmol), DCC (35 mg, 0.168 mmol), and HOBt·H$_2$O (23 mg, 0.168 mmol), and then continued the same procedure to afford **48** (22.5 mg, 31%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.56–7.50 (m, 1H), 7.40–7.22 (m, 2H), 7.18–7.08 (m, 1H), 7.00–6.89 (m, 2H), 4.93 (s, 2H), 4.60 (s, 2H), 3.67 (s, 2H), 1.22 (s, 6H); Calculated for C$_{20}$H$_{19}$ClF$_3$N$_3$O$_2$, 425.1; observed (M + H)$^+$ 426.5.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxooctahydro-1H-cyclopenta[d]pyrimidine-1-carboxamide (49)

According to the procedure for preparation of compound **21**, 2-((tert-butoxycarbonyl)amino)cyclopentanecarboxylic acid (39 mg, 0.168 mmol) was treated with 2,4-difluorobenzylamine (20 μL, 0.168 mmol), DCC (35 mg, 0.168 mmol), and HOBt·H$_2$O (23 mg, 0.168 mmol), and then continued the same procedure to afford **49** (10.5 mg, 14%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.54–7.46 (m, 1H), 7.42–7.30 (m, 1H), 7.28–7.18 (m, 1H), 7.18–7.08 (m, 1H), 6.96–6.84 (m, 2H), 5.21–5.12 (m, 1H), 4.77–4.56 (m, 4H), 3.06–2.94 (m, 1H), 2.34–2.04 (m, 2H), 1.92–1.76 (m, 2H), 1.76–1.46 (m, 2H); Calculated for C$_{21}$H$_{19}$ClF$_3$N$_3$O$_2$, 437.1; observed (M + H)$^+$ 438.6.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxo-5-phenyltetrahydropyrimidine-1(2H)-carboxamide (50)

According to the procedure for preparation of compound **21**, 3-((tert-butoxycarbonyl)amino)-2-phenylpropanoic acid (45 mg, 0.168 mmol) was treated with 2,4-difluorobenzylamine (20 μL, 0.168 mmol), DCC (35 mg, 0.168 mmol), and HOBt·H$_2$O (23 mg, 0.168 mmol), and then continued the same procedure

to afford **50** (23.6 mg, 29%). ^{1}H NMR (300 MHz, CD$_3$OD): δ (ppm) 7.52–7.42 (m, 1H), 7.40–7.20 (m, 6H), 7.12–7.06 (m, 2H), 7.06–6.90 (m, 2H), 5.22–5.14 (m, 1H), 5.00–4.90 (m, 1H), 4.82–4.64 (m, 2H), 4.14–4.04 (m, 1H), 4.04–3.95 (m, 1H), 3.95–3.82 (m, 1H); Calculated for C$_{24}$H$_{19}$ClF$_3$N$_3$O$_2$, 473.1; observed (M + H)$^+$ 474.6.

5-benzyl-N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (51)

According to the procedure for preparation of compound **21**, 2-benzyl-3-((tert-butoxycarbonyl)amino)propanoic acid (60 mg, 0.215 mmol) was treated with 2,4-difluorobenzylamine (31 mg, 0.215 mmol), EDC·HCl (41 mg, 0.215 mmol), HOBt·H$_2$O (33 mg, 0.215 mmol), and excess triethylamine, and then continued the same procedure to afford **51**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.41–7.36 (m, 1H), 7.36–7.23 (m, 4H), 7.23–7.17 (m, 2H), 7.15–7.07 (m, 1H), 7.04–6.96 (m, 1H), 6.88–6.74 (m, 2H), 5.03–4.95 (m, 1H), 4.76–4.70 (m, 1H), 4.70–4.55 (m, 2H), 3.64–3.55 (m, 1H), 3.45–3.36 (m, 1H), 3.00–2.85 (m, 1H), 2.74–2.62 (m, 1H); Calculated for C$_{25}$H$_{21}$ClF$_3$N$_3$O$_2$, 487.1; observed (M + H)$^+$ 488.6.

N-(3-chloro-4-fluorophenyl)-5-(4-cyclopropyl-1H-1,2,3-triazol-1-yl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (52)

2,4-difluorobenzylamine (34 mg, 0.239 mmol), EDC·HCl (46 mg, 0.239 mmol), HOBt·H$_2$O (37 mg, 0.239 mmol), and excess triethylamine was added to 2-azido-3-((tert-butoxycarbonyl)amino)propanoic acid **56** (50 mg, 0.217 mmol) in 2 mL DCM. The reaction was stirred overnight. After concentrating down, it was dissolved in 1:1 MeOH to 4 M HCl in dioxane for several hours and then dried overnight on high vacuum. Then, it was refluxed in acetonitrile at 50 °C overnight in a sealed flask with paraformaldehyde (8 mg, 0.251 mmol) and cyanuric chloride (4 mg, 0.0217 mmol). The reaction was diluted with EtOAc and washed with brine. The organic phase was concentrated down and dissolved in 3 mL DCM to react with phenyl (3-chloro-4-fluorophenyl)carbamate. After stirring overnight, the reaction was diluted with EtOAc and washed with 2 N HCl, saturated NaHCO$_3$, and brine. The azido-intermediate, *5-azido-N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide* **60** (10.0 mg, 11%) was obtained after HPLC separation. Calculated for C$_{18}$H$_{14}$ClF$_3$N$_6$O$_2$, 438.79; observed (M + H)$^+$ 439.5.

Ethynylcyclopropane (1 mg, 0.0137 mmol), CuI (1 mg, 0.00571 mmol), and triethylamine was added to the azido-intermediate **60** (5 mg, 0.0114 mmol) prepared above in 1 mL THF under argon. After stirring overnight, the reaction was concentrated down and purified by HPLC to afford **52**. ^{1}H NMR (300 MHz, CDCl$_3$): δ (ppm) 7.54 (s, 1H),

7.50–7.44 (m, 1H), 7.38–7.28 (m, 1H), 7.20–7.12 (m, 1H), 7.06–6.98 (m, 1H), 6.88–6.76 (m, 2H), 5.16-5.06 (m, 2H), 4.83–4.48 (m, 4H), 4.30–4.12 (m, 1H), 1.98–1.85 (m, 1H), 0.98–0.90 (m, 2H), 0.80–0.72 (m, 2H); Calculated for $C_{23}H_{20}ClF_3N_6O_2$, 504.1; observed $(M + H)^+$ 505.6.

N-(3-chloro-4-fluorophenyl)-5-(2,4-difluorobenzamido)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (53)

Triphenylphosphine (32 mg, 0.123 mmol) was added to the azido-intermediate **60** prepared in the procedure for **52** (18 mg, 0.0411 mmol) in 2 mL THF. Water was added until the solution became turbid (about 1.8 mL). THF was added dropwise until the solution became clear again. After stirring overnight, the reaction was diluted in EtOAc and washed with brine. The organic phase was purified by HPLC to afford the reduced amine intermediate **61**, 5-amino-N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahy-dropyrimidine-1(2H)-carboxamide. 2,4-difluorobenzoyl chloride (2 μL, 0.0121 mmol) was added to **61** (5 mg, 0.0121 mmol) in 1:1 EtOAc (1 ml) to saturated aqueous NaHCO3 (1 mL). After stirring overnight, the organic phase was purified by HPLC to afford **53**. ^{1}H NMR (300 MHz, CDCl3): δ (ppm) 8.08–7.97 (m, 1H), 7.40–7.30 (m, 1H), 7.25–7.19 (m, 1H), 7.07–6.76 (m, 5H), 5.03–4.90 (m, 2H), 4.85–4.77 (m, 1H), 4.74–4.64 (m, 1H), 4.56–4.44 (m, 2H), 3.30–3.20 (m, 1H); Calculated for $C_{25}H_{18}ClF_5N_4O_3$, 552.1; observed $(M + H)^+$ 553.7.

N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-5-(4-methylphenylsulfonamido)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (54)

According to the procedure for preparation of compound **53**, the reduced amino intermediate **61** (5 mg, 0.0121 mmol) was treated with 4-toluenesulfonyl chloride (3 mg, 0.0121 mmol) to afford **54**. ^{1}H NMR (300 MHz, CDCl3): δ (ppm) 7.96–7.90 (m, 1H), 7.56–7.44 (m, 2H), 7.37–7.26 (m, 2H), 7.24–7.18 (m, 2H), 7.08–7.00 (m, 1H), 6.86–6.74 (m, 2H), 5.02–4.94 (m, 1H), 4.71–4.76 (m, 1H), 4.58–4.53 (m, 2H), 4.18–4.10 (m, 1H), 3.70–3.62 (m, 1H), 3.55–3.43 (m, 1H), 2.71 (s, 3H); Calculated for $C_{25}H_{22}ClF_3N_4O_4S$, 566.1; observed $(M + H)^+$ 567.7.

5-acrylamido-N-(3-chloro-4-fluorophenyl)-3-(2,4-difluorobenzyl)-4-oxotetrahydropyrimidine-1(2H)-carboxamide (55)

According to the procedure for preparation of compound **53**, the reduced amino intermediate **61** (5 mg, 0.0121 mmol) was treated with acryloyl chloride (1 μL, 0.0121 mmol) to afford **55**. ^{1}H NMR (300 MHz, CDCl3): δ (ppm) 7.55–7.50

(m, 1H), 7.36–7.18 (m, 2H), 7.06–6.98 (m, 1H), 6.88–6.76 (m, 2H), 6.37–6.28 (m, 1H), 6.24–6.13 (m, 1H), 5.76–5.70 (m, 1H), 4.92 (s, 2H), 4.79–4.70 (m, 1H), 4.60–4.49 (m, 2H), 4.41–4.32 (m, 1H), 3.22–3.13 (m, 1H); Calculated for $C_{21}H_{18}ClF_3N_4O_3$, 466.1; observed $(M + H)^+$ 467.6.

Biological evaluation

Materials

AML12HBV10, AML12HBV_DE11, and HepDES19 cells are immortalized mouse hepatocyte (AML12)- and human hepatoma cell (HepG2)-derived stable cell lines supporting the replication of a stably-transfected envelope protein-deficient HBV genome in a tetracycline-inducible manner [38, 39]. These cell lines were maintained in DMEM/F12 medium (Corning) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 μg/ml tetracycline and 200 μg/ml G-418. When cultured in medium without tetracycline, HBV pgRNA transcription will be activated and viral DNA replication occurs subsequently. **ENAN-34017** was synthesized in house [34]. **Bay 41-4109** is a gift from Dr. Lai Wei at Peking University, Beijing China. Entecavir is a gift from Dr. William S. Mason at Fox Chase Cancer Center, Philadelphia.

Antiviral and cytotoxicity assays in AML12HBV10 cells

The cells were seeded into 96-well plates at a density of 2×10^4 cells per well and cultured in the absence of tetracycline. One day after seeding, cells were mock treated or treated with a serial twofold dilution of compound, ranging from 10 to 0.08 μM, for 48 h and lysed by addition of 100 μl per well of lysis buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, and 1% NP-40. Half of the lysate was added to DNA denaturing solution containing 1.5M NaCl and 1M NaOH. After 5 min of incubation at room temperature, 100 μl of neutralization solution containing 1.5M NaCl, 1M Tris-HCl (pH 7.4) was added. Using a 96-well dot-blot manifold (Bio-Rad), the lysates were applied to a Hybond-N+ membrane (Amersham). HBV DNA in the cell lysates was detected by hybridization with alpha-^{32}P-UTP-labeled (800 Ci/mmol, PerkinElmer) riboprobe specific for HBV minus strand DNA. After overnight incubation, membrane was washed twice, 1 h each, with buffer containing 0.1X SSC and 0.1% SDS at 65 °C, and exposed to a phosphoimager screen (GE Healthcare). Quantification done by QuantityOne software was used to determine the concentration that reduces the amount of HBV DNA by 50% (EC_{50}). To determine the cytotoxicity, the cells were treated with a serial 2-fold dilution of compound, ranging from 50 to 1.56 μM, for 48 h under the same culture condition for the antiviral assay.

The cell viability was inspected under microscopy and quantified by a MTT assay (Sigma) and expressed as the concentration of compound that reduced the viability of the cells by 50% (CC_{50}).

Antiviral and cytotoxicity assays in HepDES19 cells

For antiviral activity assay, HepDES19 cells were seeded into 24-well plates and cultured in the absence of tetracycline for 2 days. The cells were then mock treated or treated with a serial twofold dilution of compound for an additional 4 days. Cytoplasmic HBV core DNA were extracted and quantified by a qPCR assay as previously described. The antiviral activity (EC_{50}) was determined from biologically triplicated experiments by regression method of GraphPad Prism. To determine the cytotoxicity, HepDES19 cells seeded in 96-well plates were treated with a serial threefold dilution of compound, ranging from 30 to $0.12\,\mu M$, for 4 days under the same culture condition for the antiviral assay. The cell viability was inspected under microscopy and CC_{50} value was determined by a MTT assay.

Particle gels assay

AML12HBV_DE11 cells were seeded into 24-well plates and cultured in the absence of tetracycline for 6 h and then mock treated or treated with compounds at desired concentrations for an additional 30 h. The cells were lysed by a lysis buffer containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 mM NaCl, and 0.5% NP-40. Cell debris was removed by centrifugation at $12,000 \times g$ for 10 min and the lysates were subjected to electrophoresis through native 1.8% agarose gels. HBV capsids were transferred onto a Hybond N+ membrane (Amersham). After fixing the membrane in 2.5% paraformaldehyde and then in 1:1 methanol:PBS, membrane was blocked with 5% milk in TBST for 2 h at room temperature. Capsids were detected with an antibody against HBV Cp (Santa Cruz, Cat. No. sc-52406). Capsid-associated HBV DNA was detected by hybridization with an α-32P-UTP (800 Ci/mmol, PerkinElmer) labeled full-length riboprobe specific for HBV minus strand.

Western blot assay

AML12HBV_DE11 cells were lysed by 1× LDS loading buffer (Invitrogen, catalog No. NP0007). Cell lysate was boiled at 100 °C for 20 min and resolved in a NuPAGE 12% Bis-Tris protein gel (Invitrogen, catalog No. NP0342PK2), using MOPs running buffer (Genscript, catalog No. M00138) and then transferred onto a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher, catalog No.

IB24001). The membrane was probed with a rabbit polyclonal antibody against C-terminal 14 amino acid peptide of HBV Cp and he bound antibody was revealed by IRDye secondary antibodies and imaged in the LI-COR Odyssey system (LI-COR).

Compliance with ethical standards

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