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NOTE

# Synthesis, structures and anti-HBV activities of derivatives of the glutarimide antibiotic cycloheximide

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Cycloheximide (CHX) (Figure 1), produced by *Streptomyces griseus*, is a heterocyclic glutarimide antibiotic. It inhibits protein synthesis in most eukaryotic cells and is a well-known tool commonly used in biomedical research.[1] CHX has been reported to significantly inhibit both simian virus 40T antigen and cellular DNA synthesis in CV-1 cells.[2] Research efforts also showed that CHX inhibited RNA production of influenza A,[3] reovirus[4] and arbovirus,[5] and also inhibited hepatitis A virus replication.[6] Recent reports have shown that CHX contributes to the control of human immunodeficiency virus (HIV) by modifying viral protein ratios.[7,8] This observation indicates that CHX may represent an anti-HIV candidate. In this study, the inhibitory activity of CHX against hepatitis B virus (HBV) in 2.2.15 cells was investigated. The results showed that the anti-HBV activity of CHX ($IC_{50}$=45.5 μg ml$^{-1}$) was weaker than that of 3TC (Lamivudine) (Table 1). Dehydration of CHX resulted in the formation of two compounds (**1a** and **1b**), which were separated by high pressure liquid chromatography. Compounds **1a** and **1b** significantly inhibited DNA replication of HBV and the $IC_{50}$ values were 1.67 and 2.14 μg ml$^{-1}$, respectively, with high selectivity indexes (93.0 and 31.1, respectively). The structure of **1a** was elucidated by X-ray diffraction, and that of **1b** was confirmed as the 3″ *S* epimer of **1a** by CD spectroscopy.

Compounds **1a** and **1b** were produced as a mixture from CHX by dehydrating in dichloromethane with $BF_3$ $OEt_2$ as the catalyst. Schaeffer and Jain[9] have predicted that the methyl groups in CHX's dehydration product (often known as anhydrocycloheximide) exist as a mixture of both *cis* and *trans* forms. However, no previous study has confirmed whether the geometric structure of the C=C bond in anhydrocycloheximide is *Z* or *E*. The stereo structure (Figure 2) of **1a** was determined by X-ray diffraction, in which C-3″ (C10 in Figure 2b) was found to be *R*, and the double bond between C-2′ and C-1″ (C6 and C4 in Figure 2b) was the *E* configuration. Crystallographic data for the structure of **1a** have been deposited in the Cambridge Crystallographic Data Center with deposition no. CCDC719670.

The C-3″ of CHX was the *S* configuration, whereas the configuration of C-3″ of **1a** was *R*. Consequently, we deduced that **1b** must be the epimer of **1a** with C-3″ in the *S* configuration based on $^1$H NMR, $^{13}$C NMR, MS, IR and NOE spectra. In the NOE experiments, irradiation of 1′-H of **1b** enhanced the intensity of 6″-$H_\beta$, and the irradiation of 1′-H of **1a** also enhanced the intensity of 6″-$H_\beta$. Moreover, irradiation of 2′-H of compound **1a** or **1b** enhanced the intensity of 1′-H; however, no enhancement of intensity of 6″-H of **1a** or **1b** was observed. The NOE data for **1b** and **1a** indicated that the configuration of the double bond of **1b** was the same as that of **1a**, which was the *E* configuration.

To confirm that **1b** is the 3″ *S* configuration, CD spectra of compounds **1a** and **1b** were acquired (Figure 3). The CD spectra of α, β-unsaturated ketones usually show three CD bands above 180 nm. The two C=C $\pi \rightarrow \pi^*$ bands (200−260 nm) of **1a** and **1b** were similar, but the C-2″ keto $n \rightarrow \pi^*$ bands (260−400 nm) of **1a** and **1b** were opposite. Clearly, **1a** showed a negative Cotton effect, whereas **1b** showed a positive Cotton effect. Both maximum absorption wavelengths of **1a** and **1b** were near 320 nm (**1a**: 327.5 nm, $\Delta\varepsilon = -0.88$ cm$^2$ mmol$^{-1}$; **1b**: 323 nm, $\Delta\varepsilon = 0.44$ cm$^2$ mmol$^{-1}$). The difference in the CD behavior of the C-2″ keto originated from the different chirality of the C-3″. In conclusion, the data showed that **1b** is 4-[2′-(3″(*S*),5″(*S*)-3″,5″-dimethyl-2″-oxocyclohexylidene)ethyl] piperidine-2,6-dione. During the dehydration process of CHX, keto-enol tautomerization involving the C-2″ keto and the C-3″ proton leads to epimerization at the C-3″. On the basis of quantitative analysis with HPLC, the production of **1a** was ∼1.8 times greater than that of **1b**. Consequently, **1a** seems to be more stable than does **1b**. The total energies of compounds **1a** and **1b** were compared on the basis of calculations using SYBYL (version 7.35, Tripos, St Louis, MO, USA). The results support our hypothesis as the calcd total energy for **1a** was −6.053 kcal mol$^{-1}$ and that of **1b** was −5.747 kcal mol$^{-1}$.

Alkylation of the nitrogen atom of the glutarimide ring of **1a** afforded compounds **2** and **3**. Reduction of the keto group of **1a** produced compound **4** (Scheme 1). Compounds **2**, **3** and **4** did not inhibit the growth of HBV.

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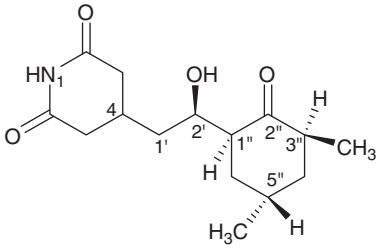


**Figure 1** Structure of cycloheximide (CHX).

**Table 1 Anti-HBV activities of CHX analogs**

| *Compounds* | *CHX* | ***1a*** | ***1b*** | ***2*** | ***3*** | ***4*** | ***3TC***[a] |
|---|---|---|---|---|---|---|---|
| $IC_{50}(\mu g\,ml^{-1})$ | 45.5 | 1.67 | 2.14 | >200 | >200 | >200 | 0.89 |
| SI[b] | 4.4 | 93.0 | 31.1 | / | / | / | 1315 |

Abbreviations: CHX, cycloheximide; HBV, hepatitis B virus; SI, selective index.
[a]3TC: Lamivudine, an antiviral agent used as positive control.
[b]$SI=TC_{50}/IC_{50}$.

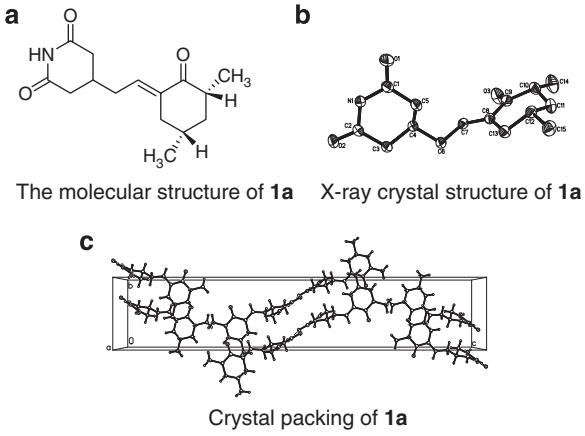



**Figure 2** X-ray structures of **1a**. (**a**) The molecular structure of **1a**. (**b**) X-ray crystal structure of **1a**. (**c**) Crystal packing of **1a**.

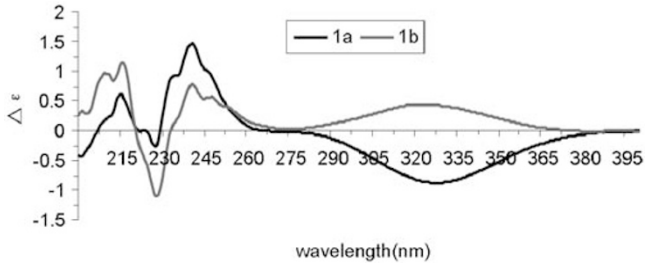
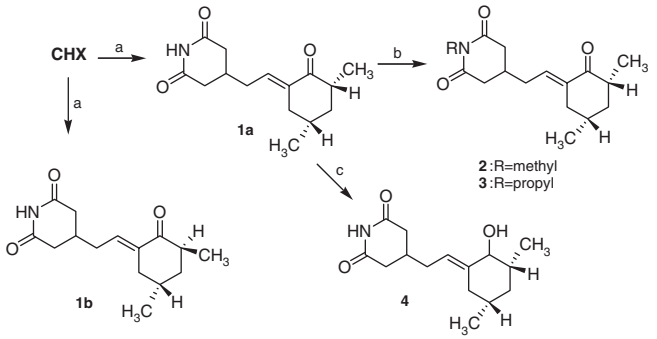



**Figure 3** CD spectra of **1a** and **1b** (in chloroform, $c=3.8\times10^{-3}$ M, at 25 °C).

In summary, CHX is a weak inhibitor of HBV in 2.2.15 cells. Under acid-catalyzed dehydration conditions in dichloromethane, the stereo configuration of the C-3″ methyl group in the cyclohexanone ring of CHX was epimerized and the two epimers **1a** and **1b** were formed. Both **1a** and **1b** showed much stronger anti-HBV activities (more than 20-fold higher) and higher selectivity indexes than did CHX. The absolute configuration of compound **1a** was determined by X-ray diffraction, and the structure of **1b** was determined on the basis of $^1$H NMR, $^{13}$C NMR, NOE and CD spectra. Alkylation of the nitrogen atom of the glutarimide ring of **1a** and reduction of the keto group of **1a** lead to the loss of anti-HBV activity. These results showed that the double bond between C-2′ and C-1″, the NH proton and the 2″ carbonyl group in the structure might be essential for anti-HBV activity.




**Scheme 1** Reagents and conditions: (**a**) $BF_3$ $OEt_2$, $CH_2Cl_2$; (**b**) $CH_3I$ or $CH_3CH_2CH_2I$, $K_2CO_3$, $CH_3CN$, 65 °C; (**c**) $Zn(BH_4)_2$-DME solution, DME. DME, 1,2-dimethoxyethane.

## EXPERIMENTAL SECTION

### General methods

Melting points were determined using an $X_6$ microscope melting point apparatus and were uncorrected. $^1$H NMR, $^{13}$C NMR spectra and NOE data were recorded on a Varian Mercury-400 spectrometer (Varian, Beijing, China). HRMS spectra were recorded on an AccuTOF CS mass spectrometer (AccuTOF, Jeol Ltd, Beijing, China). HPLC was performed using a Shimadzu LC-10Avp with a SPD-10Avp UV detector (Shimadzu, Beijing, China) and a Class VP 6.x Workstation (Shimadzu). The column used was a Diamonsil C18 5-μm 250×4.6 mm column (Dikma Technologies, Beijing, China). Preparative liquid chromatography was performed using a Shimadzu LC-6AD *vp* liquid chromatograph instrument (Shimadzu) with a SPD-10Avp UV detector and Class VP 6.x workstation. The column used was a Shim-pack PREP-ODS 20 mm ID ×25 cm column (Shimadzu) with a 100-Å pore diameter and 15-μm particle diameter. Optical rotations were measured on a Perkin Elmer Model 341 LC parameter (Perkin Elmer, Shanghai, China). X-ray data were collected on a MAC DIP-2030K diffractometer. Circular dichroism spectra were recorded on a Jasco J-815 CD spectropolarimeter (Jasco, Tokyo, Japan).

### 4-[2′-(3″(*R*),5″(*S*)-3″,5″-dimethyl-2″-oxocyclohexylidene)ethyl] piperidine-2,6-dione (1a) and 4-[2′-(3″(*S*),5″(*S*)-3″,5″-dimethyl-2″-oxocyclohexylidene)ethyl]piperidine-2,6-dione (1b)

$BF_3$ $OEt_2$ (1.76 ml, 1.4 mmol) was added slowly to an ice-salt cold solution of CHX (2.0 g, 7 mmol) in 20 ml of dichloromethane. After stirring for 5 min, the salt ice-bath was withdrawn and stirring was continued for 3 h at room temperature. The ice-bath was used at the end of the stirring period. The solution was quenched with 20 ml of saturated $NaHCO_3$ solution when the temperature of the solution reached 0 °C. The water layer was extracted with dichloromethane (20 ml×3). The combined organic layer was washed with 60 ml of saturated NaCl and 60 ml of water and then dried over anhydrous magnesium sulfate. The solution was filtered to remove the precipitate and the filtrate was concentrated *in vacuo*. The residue was purified by chromatography using a silica gel column (100:15 dichloromethane/acetone) to afford a mixture of the compounds **1a** and **1b** (1.10 g, yield 55%) as a white powder.

Compounds **1a** and **1b** were separated by preparative liquid chromatography. The chromatography was performed on a Shim-pack PREP-ODS at 25 °C with a mobile phase of water–methanol 50:50 (v/v). The flow rate was 10 ml min$^{-1}$, and detection was achieved by monitoring the UV absorbance at 231 nm. The sample was dissolved in dimethyl sulfoxide, and sample-loading volumes were 100 μl. The separated solution was concentrated until no

methanol existed and was then dried by vacuum freeze drying. Compounds **1a** and **1b** were isolated and both appeared as a white powder.

Physical data for compound **1a**: m.p. 130–131 °C; $[\alpha]_D^{20}$ =−30.9 °C (*c*=1.25, $CHCl_3$); IR: 3189, 2930 (NH), 1712 (C=O), 1682 (C=O) $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 1.05 (3H, d, *J*=5.6 Hz, 5″-Me), 1.13 (3H, d, *J*=6.8 Hz, 3″-Me), 1.22–1.32 (1H, m, 4″-$H_\alpha$), 1.84–1.93 (2H, m, 5″-H, 6″-$H_\alpha$), 1.96–2.01 (1H, m, 4″-$H_\beta$), 2.16–2.25 (2H, m, 1′-H), 2.27–2.38 (4H, m, 3″-H, 4-H, 3-$H_\alpha$, 5-$H_\alpha$), 2.67–2.73 (3H, m, 6″-$H_\beta$, 3-$H_\beta$, 5-$H_\beta$), 6.35 (1H, t, *J*=8.0 Hz, =CH), 7.85 (1H, br, NH); $^{13}C$ NMR (100 MHz, $CDCl_3$): 15.7 (3″-Me), 22.1 (5″-Me), 30.1 (C-4), 30.3 (C-5″), 32.4 (C-1′), 36.2 (C-6″), 37.4 (C-3), 37.4 (C-5), 40.7 (C-4″), 44.1 (C-3′), 131.6 (C-2′), 139.3 (C-1″), 171.5 (C-2, C-6), 203.2 (C-2″); MS ($ESI^+$) *m/z*: 286.4 $[M+Na]^+$, HRMS ($ESI^+$) *m/z*: 286.14183 $[M+Na]^+$, calcd mass for $C_{15}H_{21}NO_3Na$: 286.14191.

Physical data for compound **1b**: m.p. 118–120 °C; $[\alpha]_D^{20}$ =+45.7 °C (*c*=1.03, $CHCl_3$); IR: 3201, 2927 (NH), 1721 (C=O), 1678 (C=O) $cm^{-1}$; 1H NMR (400 MHz, $CDCl_3$) $\delta$: 1.04 (3H, d, *J*=6.4 Hz, 5″-Me), 1.12 (3H, d, *J*=7.2 Hz, 3″-Me), 1.64–1.77 (2H, m, 4″-H), 2.01–2.11 (2H, m, 4-H, 6″-$H_\alpha$), 2.19–2.21 (2H, m, 1′-H), 2.28–2.31 (3H, m, 5″-H, 3-$H_\alpha$, 5-$H_\alpha$), 2.52–2.59 (2H, m, 3″-H, 6″-$H_\beta$), 2.65–2.70 (2H, m, 3-$H_\beta$, 5-$H_\beta$), 6.40 (1H, t, *J*=7.6 Hz, =CH ), 8.47 (1H, br, NH); $^{13}C$ NMR (100 MHz, $CDCl_3$): 17.1 (3″-Me), 21.1 (5″-Me), 25.9 (C-4), 30.1 (C-5″), 32.4 (C-1′), 34.8 (C-6″), 37.4 (C-3, C-5), 38.2 (C-4″), 40.4 (C-3′), 132.5 (C-2′), 138.4 (C-1″), 171.4 (C-2, C-6), 204.0 (C-2″); MS ($ESI^+$) *m/z*: 286.4 $[M+Na]^+$, HRMS ($ESI^+$) *m/z*: 286.14111 $[M+Na]^+$, calcd mass for $C_{15}H_{21}NO_3Na$: 286.14191.

### *N*-methyl-4-[2′-(3″(*R*),5″(*S*)-3″,5″-dimethyl-2″-oxocyclohexylidene)ethyl]piperidine-2,6-dione (2)

Compound **1a** (50 mg, 0.19 mmol), iodomethane (97 mg, 0.57 mmol) and anhydrous potassium carbonate (32 mg, 0.23 mmol) were added to 3 ml of acetonitrile and stirred at 65 °C for 10 h. The solution was filtered to remove potassium carbonate and the filtrate was concentrated. The residue was dissolved in 10 ml of ethyl acetate and washed with water. The organic layer was dried over anhydrous sodium sulfate. The solution was filtered to remove any solids and the filtrate was concentrated under vacuum. The residue was purified by chromatography using a silica gel column (100:7 dichloromethane/methanol) to obtain the title compound **2** (48.5 mg, yield 92%) as a white solid.

Physical data for compound **2**: m.p. 47–49 °C; $[\alpha]_D^{20}$ =−24.9 °C (*c*=0.25, $CHCl_3$); IR: 1720 (C=O), 1671 (C=O) $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 1.05 (3H, d, *J*=5.6 Hz, 5″-Me), 1.13 (3H, d, *J*=6.8 Hz, 3″-Me), 1.23–1.32 (1H, m, 4″-$H_\alpha$), 1.87–1.92 (2H, m, 5″-H, 6″-$H_\alpha$), 1.96–2.01 (1H, m, 4″-$H_\beta$), 2.14–2.26 (3H, m, 1′-H, 4-H), 2.31–2.39 (3H, m, 3″-H, 3-$H_\alpha$, 5-$H_\alpha$), 2.70 (1H, d, *J*=13.2 Hz, 6″-$H_\beta$), 2.80 (2H, d, *J*=17.2 Hz, 3-$H_\beta$, 5-$H_\beta$), 3.14 (3H, s, N-Me), 6.35 (1H, t, *J*=7.6 Hz, =CH); $^{13}C$ NMR (100 MHz, $CDCl_3$): 15.7 (3″-Me), 22.1 (5″-Me), 26.4 (N-C), 29.2 (C-4), 30.3 (C-5″), 32.6 (C-1′), 36.2 (C-6″), 38.4 (C-3), 38.5 (C-5), 40.8 (C-4″), 44.0 (C-3″), 131.8 (C-2′), 139.1 (C-1″), 171.8 (C-2, C-6), 203.5 (C-2″); MS ($ESI^+$) *m/z*: 278.5 $[M+H]^+$ and 300.5 $[M+Na]^+$, HRMS ($ESI^+$) *m/z*: 300.15629 $[M+Na]^+$, calcd mass for $C_{16}H_{23}NO_3Na$: 300.15756.

### *N*-propyl-4-[2′-(3″(*R*),5″(*S*)-3″,5″-dimethyl-2″-oxo-cyclohexylidene)-ethyl]piperidine-2,6-dione (3)

The title compound was obtained from **1a** using a procedure similar to that used to prepare compound **2**. The yield was 17% as yellow syrup.

Physical data for compound **3**: $[\alpha]_D^{20}$ =−20.6 °C (*c*=0.56, $CHCl_3$); IR: 1725 (C=O), 1676 (C=O) $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 0.88 (3H, t, *J*=7.2 Hz, N-γ-Me), 1.04 (3H, d, *J*=6.0 Hz, 5″-Me), 1.12 (3H, d, *J*=6.8 Hz, 3″-Me), 1.25–1.31(1H, m, 4″-$H_\alpha$), 1.51(1H, sext, *J*=7.2 Hz, N-β-$CH_2$), 1.86–1.92 (2H, m, 5″-H, 6″-$H_\alpha$), 1.96–1.99 (1H, m, 4″-$H_\beta$), 2.11–2.27 (3H, m, 1′-H, 4-H), 2.31–2.39 (3H, m, 3-$H_\alpha$, 5-$H_\alpha$, 3″-H), 2.70 (1H, d, *J*=13.2 Hz, 6″-$H_\beta$), 2.77 (2H, d, *J*=16.8 Hz, 3-$H_\beta$, 5-$H_\beta$), 3.71 (2H, t, *J*=7.2 Hz, N-α-$CH_2$), 6.34 (1H, t, *J*=7.6 Hz, =CH); $^{13}C$ NMR (100 MHz, $CDCl_3$): 11.3 (N-γ-C), 15.7 (3″-Me), 21.2 (N-β-C), 22.1 (5″-Me), 29.2 (C-4), 30.3 (C-5″), 32.5 (C-1′), 36.2 (C-6″), 38.5 (C-3), 38.6 (C-5), 40.8 (C-4″), 41.2 (N-α-C),44.0 (C-3″), 131.9 (C-2′), 139.1 (C-1″), 171.6 (C-2, C-6), 203.2 (C-2″); MS ($ESI^+$) *m/z*: 306.5 $[M+H]^+$, 328.5 $[M+Na]^+$, HRMS ($ESI^+$) *m/z*: 328.18554 $[M+Na]^+$, calcd mass for $C_{18}H_{27}NO_3Na$: 328.18886.

### 4-[2′-(3″(*R*),5″(*S*)-3″,5″-dimethyl-2″-hydroxyl-cyclohexylidene)ethyl]piperidine-2,6-dione (4)

To a solution of **1a** (50 mg, 0.19 mmol) in 3 ml of 1,2-dimethoxyethane (DME), $Zn(BH_4)_2$-DME (4 ml, prepared by the method described by Gensler *et al.*[10]) was added slowly. After stirring at room temperature for 24 h, 4 ml of water and 1 ml of glacial acetic was added to the reaction mixture. The solution was filtered to remove the precipitate and the filtrate was concentrated under vacuum. The residue was purified by chromatography using a silica gel column (150:10 dichloromethane/methanol). The title compound was collected as a white solid (42 mg, yield 84%).

Physical data for compound **4**: m.p. 39–41 °C; $[\alpha]_D^{20}$ =−15.5 °C (*c*=0.47, $CHCl_3$); IR: 3476 (OH), 3198, 3095 (NH), 1681 (C=O) $cm^{-1}$; $^1H$ NMR (400 MHz, $CDCl_3$) $\delta$: 0.89–0.92 (m, 4″-$H_\alpha$), 0.94 (3H, d, *J*=6.0 Hz, 5″-Me), 1.07 (3H, d, *J*=6.4 Hz, 3″-Me), 1.35–1.44 (3H, m, 3″-H, 5″-H, 6″-$H_\alpha$), 1.56 (s, OH), 1.72–1.76 (1H, m, 4″-$H_\beta$), 2.18–2.22 (2H, m, 4-H, 1′-H), 2.28–2.34 (2H, m, 3-$H_\alpha$, 5-$H_\alpha$), 2.52 (d, *J*=12.0 Hz, 6″-$H_\beta$), 2.67–2.72 (2H, m, 3-$H_\beta$, 5-$H_\beta$), 3.56 (1H, d, *J*=10.0 Hz, 2″-H), 5.42 (1H, t, *J*=6.8 Hz, =CH), 7.81 (1H, br, NH); $^{13}C$ NMR (100 MHz, $CDCl_3$): 19.1 (3″-Me), 22.1 (5″-Me), 30.9 (C-4), 31.8 (C-1″), 33.4 (C-5″), 36.5 (C-6″), 37.3 (C-3), 37.4 (C-5), 41.4 (C-3″), 42.5 (C-4″), 77.9 (C-2″), 113.1 (C-2′), 144.0 (C-1″), 172.2 (C-2, C-6); MS ($ESI^+$) *m/z* : 288.3 $[M+Na]^+$ and 248.3 $[M-H_2O]^+$, HRMS ($ESI^+$) *m/z*: 288.15935 $[M+Na]^+$, calcd mass for $C_{15}H_{23}NO_3Na$: 288.15756.

### Anti-HBV assays

2.2.15 cells in exponential growth phase were seeded into 96-well microplates. A total of $2\times10^4$ cells per well were used. Test compounds with different concentrations were added, and the cells were cultured in $CO_2$ incubators at 37 °C. The medium was exchanged every 4 days. The positive control (3TC) and untreated cell negative control groups were established. After 8 days, HBV DNA of the lysed cells was extracted. HBV DNA levels were measured using a quantitative Southern blot hybridization analysis approach.

### Cytotoxicity assay

Cytotoxicity induced by the test compounds in the cultures of 2.2.15 cells was also determined. 2.2.15 cells were seeded into 96-well microplates: $1\times10^5$ cells per ml, 100 µl per well. Cells were treated with test compounds at different concentrations. The control was untreated cells. The culture medium was exchanged every 4 days. After 8 days, cell viability levels were examined. $TC_{50}$ was defined as the concentration that inhibited 50% cellular growth in comparison with untreated controls and was calculated by Reed and Muench analyses.

## ACKNOWLEDGEMENTS

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