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Design and Synthesis of Oxymatrine Analogues Overcoming Drug Resistance in Hepatitis B Virus through Targeting Host Heat Stress Cognate 70

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Heat-stress cognate 70 (Hsc70) is a host protein required for hepatitis B virus (HBV) replication, and oxymatrine (**1**) suppresses Hsc70 expression. Taking Hsc70 as a target against HBV, 22 analogues of **1** defined with substituents at position 1, 13, or 14 were synthesized and evaluated for their activity on Hsc70 mRNA expression. The SAR revealed that (i) the oxygen atom at the 1-position was not essential, (ii) increasing electron density on the ring D reduced the activity, and (iii) introducing a proper substituent at the 13- and/or 14-position(s), especially electron-withdrawing groups, might enhance the activity. Among the analogues, **6b** possessing 13-ethoxyl afforded an increased activity in respect to **1**. Importantly, it was active for either wild-type or lamivudine-resistant HBV, as its target is host Hsc70 but not viral enzymes. LD_{50} of **6b** in mice was over 750 mg/kg in oral route. We consider compound **6b** promising for further investigation.

Introduction

Oxymatrine (**1**, Figure 1) is a quinolizidine natural drug extracted from *Sophora japonica* (Kushen). It has been used to treat hepatitis B patients in China for decades with confirmed safety,[1−3] although its mode of action against hepatitis B virus (HBV[a]) infection remains unknown. Recently, we have discovered host heat-stress cognate 70 (Hsc70) to be a key target of **1** in its action against HBV[4] and demonstrated a novel anti-HBV mechanism different from that of nucleosides with viral enzymes as target. Biological study showed that Hsc70 is a host protein required for the function of HBV replication.[5] Compound **1** significantly down-regulates host Hsc70 expression at the post transcriptional level.[4] It destabilizes Hsc70 mRNA through its 3′UTR region and therefore creates a cellular environment not supportive for HBV amplification.[4] Importantly, it is active for either wild-type HBV or those resistant to inhibitors of HBV DNA polymerase (DNAp) such as lamivudine and adefovir that induces drug-resistance in clinic.[6] As host Hsc70 was the target, chemotherapeutic pressure of compound **1** was not on viral components and therefore it inhibits viral replication with no or decreased chance of inducing drug-resistant mutations.[5] In addition, Hsc70 gene knockout showed no abnormality in mice, indicating a good safety after inhibition of Hsc70.[7]

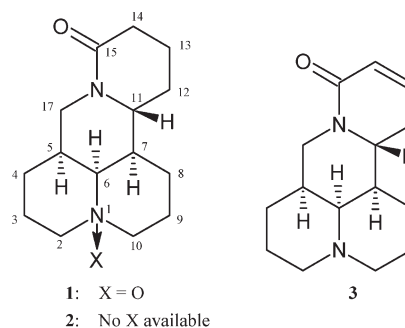

Figure 1. Chemical structures of compounds **1**−**3**.

Therefore, we consider Hsc70 a novel and potential target to discover drug candidates against HBV. In the present study, the structure−activity relationship (SAR) of compound **1** was done for the activity on Hsc70 mRNA expression with **1** as the lead. As the nitrogen atom or carbonyl at the 1- or 15-position might both be essential for the activity, the SAR analysis was first focused on the influence of the oxygen atom at the position 1 and substitutions at α- or β-position (13-, or 14-position) of the carbonyl group on the ring D. On the basis of this strategy, 22 derivatives with various substituents at 1, 13- and/or 14-position(s) were designed, obtained, and evaluated for their effect in Hsc70 mRNA expression. Anti-HBV activity was measured in parallel.

Here, we describe the analogues of compound **1** for their semisynthesis, Hsc70 expression regulation, SAR analysis, in vitro anti-HBV effect for both wild-type and drug-resistant HBV, and toxicity in vivo as well as mode of action.

Chemistry

Compound **1**, matrine (**2**), and sophocarpine (**3**) (Figure 1) with purity over 98.5% were purchased from the National

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[a] Abbreviations: Hsc70, heat-stress cognate 70; HBV, hepatitis B virus; HepG2.2.15, HepG2 cells transfected with full genome of HBV; SAR, structure−activity relationship; LRS, lamivudine-resistance strains; PFA, foscarnet; DNAp, DNA polymerase; BUN, blood urea nitrogen; CRE, creatine; GOP, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase.

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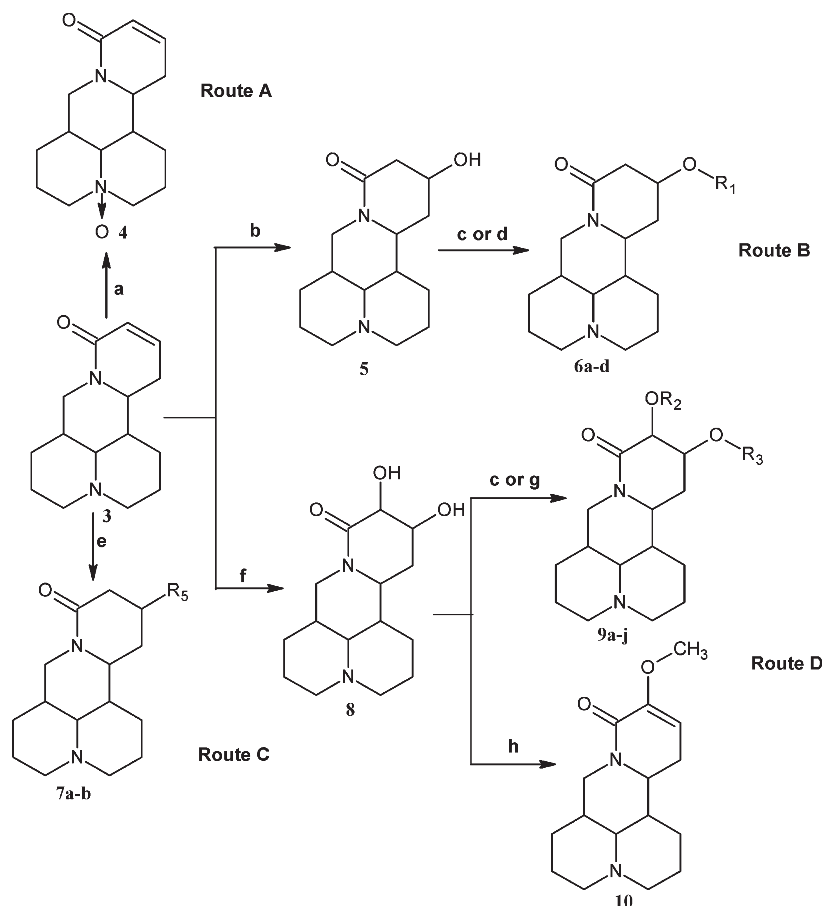
Scheme 1. Synthetic Routes of the Study Compounds[a]



[a] Reagents and conditions for the chemical synthesis: (a) H_2O_2 (30%), 50 °C, 16 h; (b) KOH/H_2O, reflux, 8 h; (c) halogenated hydrocarbon, 50% KOH/H_2O, rt, 8 h; (d) benzoyl choride, KOH powder, CH_2Cl_2, rt, 4 h; (e) sodium alkoxide or DBU, 24 h; (f) $KMnO_4$, 0 °C, 2 h; (g) appropriate acyl chloride, CH_2Cl_2, rt, 1−24 h; (h) CH_3I, 50% KOH/H_2O, acetone, rt, 8 h.

Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Twenty analogues were semisynthesized with commercial available **3** as the starting material as described in Scheme 1 that includes four synthetic methods (routes A, B, C, and D).

The desired oxysophocarpine **4** (route A) was prepared for a selective oxidation reaction of **3** with a yield of 66% in which hydrogen peroxide (30%) was used as an oxidant and water as the solvent. The key intermediate **5** (route B) was gotten by the electrophilic addition of **3** with water in the presence of KOH and then reacted with methyl iodine, ethyl iodine, or benzyl bromide respectively in 50% KOH aqueous and converted into **6a**−**c** in a yield of 20−57%. Compound **6d** was obtained through esterification of **5** with benzoyl chloride in the presence of KOH powder in a yield of 37%.[8]

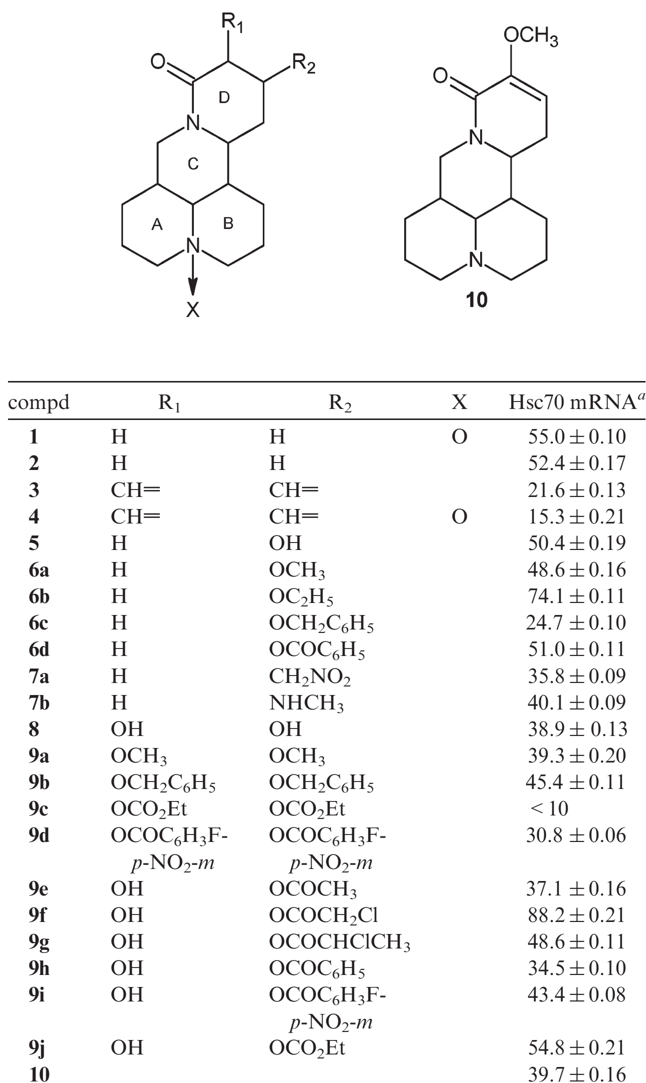
Compounds **7a**−**b** (route C) were synthesized through Michael addition of **3** with methylamine or nitromethane, respectively, using sodium alcoholate or DBU as the base.[9,10] Another key intermediate **8** was obtained via oxidation reaction using $KMnO_4$ as an oxidizing agent and water−acetone as the solvent. Compounds **9a** and **9b** were prepared by the etherification of **8** with methyl iodine or benzyl bromide in 50% KOH aqueous with a yield of 37% and 81%, respectively. Similarly, compounds **9c**−**j** were obtained via esterification of **8** with corresponding acyl chloride in CH_2Cl_2 at room temperature.[11] During the course of the esterification, owing to the steric hindrance of carboxyl at the 15-position, the hydroxyl at the 13-position of **8** underwent esterification in priority, and thus monoester (**9e**−**j**) were obtained with a yield of 21−51%. In addition, 13,14-dihydroxyl of **8** underwent esterification simultaneously, and thus compounds (**9c**−**d**) were synthesized in the presence of KOH powder. Compound **10** was obtained through etherification of **8** with CH_3I and then elimination of H_2O in the presence of KOH using acetone as the solvent. Its structure was confirmed by ^1H NMR spectra in which the hydrogen at the 13-position was a triplet but not a singlet.

Results and Discussion

SAR Analysis for Down-Regulating Hsc70 mRNA Expression. The screening assay for Hsc70 mRNA expression was employed for the initial evaluation in human liver HepG2 cells transfected with full genome of HBV (HepG2.2.15).[4] Hsc70 mRNA expression was determined with specific real-time RT-PCR reaction. Structures of the 22 compounds and their effect on Hsc70 mRNA expression are shown in Table 1.

SAR study was initiated with the function of the oxygen atom at position 1, for which compounds **2**−**4** were purchased or semisynthesized. Oxygen atom was removed from **1**, and the resultant compound **2** kept a moderated down-regulating activity on Hsc70 expression by over 50%, almost identical to that of **1**. Introduction of an oxygen atom at the 1-position of **3**, compound **4** showed mild inhibitory activity, same as its parent **3** did. We deduce that the oxygen atom at

Table 1. Structures and Down-Regulating Hsc70 mRNA Expression of Study Compounds



compd	R_1	R_2	X	Hsc70 mRNA[a]
1	H	H	O	55.0 ± 0.10
2	H	H		52.4 ± 0.17
3	CH=	CH=		21.6 ± 0.13
4	CH=	CH=	O	15.3 ± 0.21
5	H	OH		50.4 ± 0.19
6a	H	OCH_3		48.6 ± 0.16
6b	H	OC_2H_5		74.1 ± 0.11
6c	H	$OCH_2C_6H_5$		24.7 ± 0.10
6d	H	$OCOC_6H_5$		51.0 ± 0.11
7a	H	CH_2NO_2		35.8 ± 0.09
7b	H	$NHCH_3$		40.1 ± 0.09
8	OH	OH		38.9 ± 0.13
9a	OCH_3	OCH_3		39.3 ± 0.20
9b	$OCH_2C_6H_5$	$OCH_2C_6H_5$		45.4 ± 0.11
9c	OCO_2Et	OCO_2Et		< 10
9d	$OCOC_6H_3F$-*p*-NO_2-*m*	$OCOC_6H_3F$-*p*-NO_2-*m*		30.8 ± 0.06
9e	OH	$OCOCH_3$		37.1 ± 0.16
9f	OH	$OCOCH_2Cl$		88.2 ± 0.21
9g	OH	$OCOCHClCH_3$		48.6 ± 0.11
9h	OH	$OCOC_6H_5$		34.5 ± 0.10
9i	OH	$OCOC_6H_3F$-*p*-NO_2-*m*		43.4 ± 0.08
9j	OH	OCO_2Et		54.8 ± 0.21
10				39.7 ± 0.16

[a] % of inhibition. HepG2.2.15 cells were cultured in the MEM medium and incubated respectively with compound **1** analogues (100 μg/mL) for 24 h. Down-regulation of Hsc70 mRNA expression was determined with real-time RT-PCR method. The data shown were mean ± SD of three separate experiments for % of the inhibition of Hsc70 expression.

position 1 is not an essential element to keep the compounds with potency. Therefore, the derivatives without oxygen atom at the 1-position were examined subsequently.

On the other hand, compounds **3** and **4** bearing a carbon−carbon double bond at positions 13 and 14 largely lost their activity as compared to parent **2** or **1**, respectively. Introduction of a methoxyl at the 14-position of **3** (**10**) resulted in a partially increased activity over that of parent **3**, owing to the electron-withdrawing feature of the methoxyl. It appeared that the increase of electron cloudy density on the ring D is not beneficial for the down-regulation of Hsc70 mRNA expression. Another explanation possibly contributed to the reduced activity of the compounds is the conformational alteration of the **3** after introducing a carbon−carbon double bond.[12] Compound **3** retains the same structural form of the A/B/C fragment as **2**, but the conformation with the C/D-(quasi)-*trans* ring junction (**2**) is in a tendency to convert into the (quasi)-*cis* ring junction because of the barrier to interconversion being low (Figure 2).[12d]

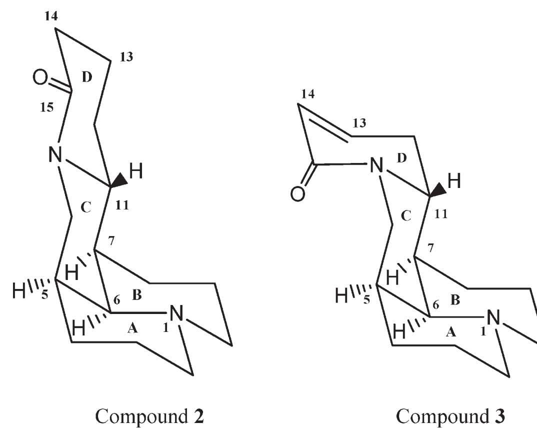


Figure 2. Conformational analysis of compound **2** and **3**. The conformational structures were drawn based on the previous reports.[12]

These factors appeared to interrupt the interactions between the compounds and speculated target(s).

Next, the SAR analysis was done with modifications at the 13-position. Attachment of a hydroxyl, methoxyl, ethoxyl, benzyloxy, benzoyloxy, nitromethyl, or methylamino at the 13-position, respectively, generated seven analogues (**5**, **6a−d**, **7a−b**). Compound **6b** possessing an ethoxyl at the 13-position afforded the most potent activity in down-regulating Hsc70 expression with 1.3-fold increase over that of **1**. The compounds bearing methoxyl (**6a**), hydroxyl (**5**), or benzoyloxy (**6d**) at position 13 afforded a moderated effect similar to that of **1**. Other compounds such as nitromethyl (**7a**), methylamino (**7b**), and benzyloxy (**6c**) showed a partial loss of the activity on Hsc70 mRNA expression. It seemed that introduction of a proper substituent at the 13-position regardless of the size of the side chains might enhance the activity of **1**.

In another variation, SAR analysis was carried out to explore the effect of the substituents at positions 13 and 14. The substituents with different sizes of hydroxyl (**8**), methoxyl (**9a**), benzyloxy (**9b**), ethoxyacyloxy (**9c**), and nitrobenzoyloxy (**9d**) were added at the positions 13 and 14 simultaneously, from which five analogues were created and tested. Compound **9b** showed a moderated activity on Hsc70 mRNA expression similar to that of **1**. Compounds **8**, **9a**, and **9c−d** lost their activity partially or completely. By attaching a hydroxyl at the 14-position and an acetoxy (**9e**), chloroacetoxy (**9f**), chloropropionyloxy (**9g**), benzoyloxy (**9h**), nitrobenzoyloxy (**9i**), or ethoxyacyloxy (**9j**) at position 13, respectively, six analogues were designed and synthesized. Compounds **9e** and **9g−j** showed an activity on Hsc70 lower than that of **1**. Compound **9f** exhibited the strongest Hsc70 down-regulatory activity among the study compounds, with 1.6-fold increase over that of **1**. Thus, attachment of the suitable substituent at the 13- and/or 14-position(s) might improve the down-regulating activity on Hsc70 mRNA expression.

Anti-HBV Effect in Vitro. To verify the anti-HBV effect of the active compounds screened through Hsc70 mRNA expression, 13 positive compounds (**6a−d**, **7a−b**, **8**, **9b**, **9e−h**, **9j**) were examined for their anti-HBV effect in the HepG2.2.15 cells using the assay described before.[13] The inhibition of HBV DNA basically agrees with the down-regulation of Hsc70 mRNA in compounds with high inhibition rate on Hsc70 (over 50% inhibition); however, when the inhibition rate for Hsc70 is low, the correlation does not stand (Table 2). The in vitro cytotoxicity (TC_{50} values) was assessed to calculate their selective index (SI). Among these analogues, SI values of compounds **6b** and **9f** were

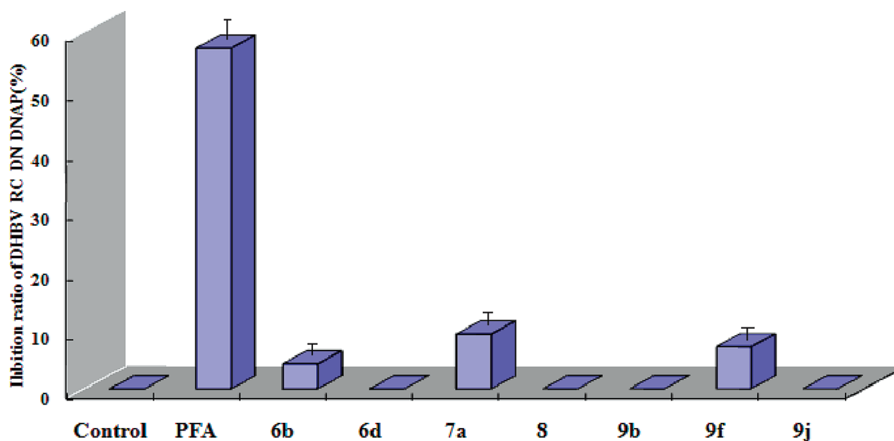


Figure 3. Effect of **6b** on DHBV DNAp. Inbihitory activity on DHBV DNAp was examined in some of the compounds (1000 μg/mL) that were active against HBV. For the experimental procedure, see Experimental Section. Values are mean ± SE of 3 separate experiments.

Table 2. Selective Index of Some Compounds against HBV in HepG2.2.15 Cells

compd	IC_{50} (μM)[a]	TC_{50} (μM)[b]	SI[c]
6a	120.91	1105.3	9.14
6b	9.55	480.75	50.34
6c	14.38	135.68	9.43
6d	16.3	236.01	14.48
7a	54.45	> 1000	> 18.36
7b	167.73	> 1000	> 5.96
8	47.14	> 1000	> 21.21
9b	2.76	37.04	13.42
9e	346.37	> 1000	2.89
9f	13.96	1104.83	79.14
9g	591.57	> 1000	1.69
9h	42.57	135.68	3.18
9j	23.88	1000	41.87
PFA	106.3	> 1000	> 9.41

[a] IC_{50}: defined as the concentration that inhibited 50% cellular HBV DNA in comparison to untreated controls in HepG2.2.15 cells. [b] TC_{50}: defined as the concentration killing 50% cells in comparison to untreated controls in HepG2.2.15 cells. [c] SI = IC_{50}/TC_{50}.

50.3 and 79.1, respectively, much higher than the others. Both of them exhibited a potential anti-HBV activity and low toxicity in HepG2.2.15 cells.

The above results suggested that down-regulation of Hsc70 expression might be the main mechanism for the study compounds in their action against HBV. To confirm the mode of action for these compounds, their inhibitory activity on HBV DNAp was examined. In the present study, isolated duck HBV (DHBV) DNAp was used for the experiment as human HBV DNAp is difficult to obtain worldwide. As foscarnet (PFA) is effective in inhibiting DHBV DNAp both in vitro and in vivo,[13] it was used as a reference drug. Among the compounds in Table 2, those with SI values over 10 were chosen for the test of their activity on DHBV DNAp. The results were basically consistent with our anticipation. As shown in Figure 3, none of these compounds showed inhibitory activity on DHBV DNAp when the concentration was as high as 1000 μg/mL. It excludes the possible HBV DNAp inhibition mechanism of these compounds and indicates the difference in anti-HBV mechanism between the known HBV DNAp inhibitors and the study compounds. Therefore, we deduce that host Hsc70 might be at least one of the key drug targets for the **1** analogues in their action against HBV. Down-regulating activity on Hsc70 mRNA expression could be an initial screen assay to discover compounds

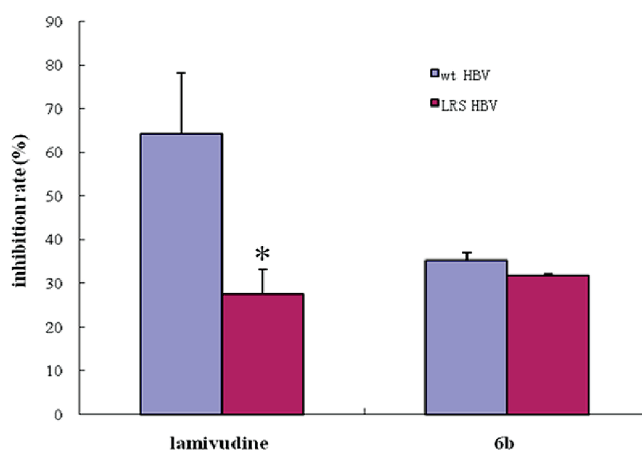


Figure 4. Antiviral effect of **6b** in lamivudine-resistance HBV. Huh-7.5 cells were transiently transfected with the full genome of wide-type or lamivudine-resistant HBV, followed by 36 h treatment with lamivudine (0.16 μg/mL) or **6b** (37 μg/mL), respectively. HBV DNA replicative intermediate level was measured using real-time PCR. The experiment was performed twice. Shown are means and SD. *, $P < 0.05$ (wild type HBV vs lamivudine-resistant HBV) in the Student *t*-test.

effective on HBV. As the drug target is a host protein but not a viral component, theoretically the advantage of these compounds is that they should be effective on both wild-type as well as drug-resistant HBV.

Effect of 6b on Lamivudine-Resistant HBV Strains. As compound **6b** afforded an anti-HBV activity (IC_{50} = 9.55 μM) greater than that of **9f** (IC_{50} = 13.96 μM), it was chosen to examine its effect on HBV strains resistant to HBV DNAp inhibitors. In this experiment, Huh-7.5 cells transiently transfected with lamivudine-resistant strains (LRS) were used. Using lamivudine as a reference drug, the wild-type strain or LRS were treated with lamivudine (0.16 μg/mL) or **6b** (37 μg/mL) respectively for 36 h, followed by examination of the level of HBV DNA replicative intermediate using real time PCR.[14] As shown in Figure 4, lamivudine suppressed the wild-type HBV replication by 64%, much higher than its inhibiting effect on the lamivudine-resistant HBV, which was only 28%. However, compound **6b** at its relatively low concentration of 37 μg/mL inhibited the drug-resistant HBV replication with a potency similar to its effect on wild-type HBV (35% vs 32%), indicating the effectiveness of **6b** on both strains. The result was consistent with that of **1** in our previous reports.[4]

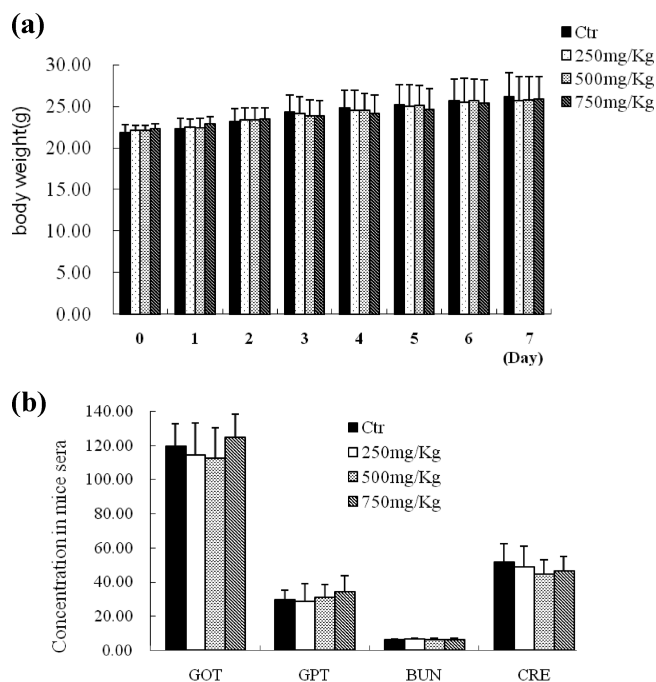


Figure 5. Safety of **6b** in mice. (A) Effect of **6b** (oral) on mice body weight. (B) Effect of **6b** on liver and kidney functions. BUN, blood urea nitrogen (mM); CRE, creatine (μM); GOP, glutamate-oxaloacetate transaminase (U/L); GPT, glutamate-pyruvate transaminase (U/L) levels in the treatment with **6b**. Presented are means and SDs.

Safety Evaluation in Vivo for 6b. As compound **6b** inhibited host Hsc70 expression, we next performed safety evaluation of **6b** for its acute toxicity in mice. Compound **6b** was given orally in a single-dosing experiment at 250, 500, or 750 mg/kg, respectively. The mice were closely monitored for 7 days. No mouse died during the 7-day observation duration, indicating that the LD_{50} of **6b** in oral route was larger than 750 mg/kg. Oral administration of **6b** at the experiment doses did not affect the body weight of the mice as well (Figure 5A). Blood samples taken at the end of the experiment were examined for liver and kidney functions. Significant abnormality was not found in blood AST, ALT, BUN, and CRE in the **6b** treatment groups, even when the dose was up to 750 mg/kg (Figure 5B). The results suggest that compounds with an action of down-regulating Hsc70 expression could be safe in vivo, consistent with the previous report in gene knockout mice.[7]

Conclusions

By using host Hsc70 as a novel target against HBV, we have designed and synthesized 22 analogues of compound **1** with modifications at positions 1, 13, and/or 14. Their biological effects on Hsc70 mRNA expression were analyzed. The SAR analysis indicated that (i) the oxygen atom at the 1-position is not an important element to keep the activity, (ii) increasing electron density on the ring D reduces the activity, and (iii) introducing suitable substitutents at position(s) 13 and/or 14, especially those with electron-withdrawing feature might enhance the down-regulatory effect on Hsc70 expression. Anti-HBV activity of the compounds was investigated in cell culture and viral enzyme systems in comparison with their effect on Hsc70 expression. Among these analogues, compounds **6b** showed a potential activity for either wild-type or lamivudine-resistant HBV; thus it had been selected as an anti-HBV drug candidate for further investigation. We consider the compounds with inhibitory effect on Hsc70 mRNA expression to be a group of novel anti-HBV compounds with an advantage of inhibiting either wild-type or drug-resistant HBV.

Experimental Section

Chemistry. Melting point (mp) was obtained with MPA 100 OptiMelt automated melting point system (Stanford Research Systems, CA, USA). ^{1}H NMR spectra analysis was performed on a Varian Inova 400 MHz spectrometer (Varian, San Francisco, CA, USA) in $CDCl_3$ or CD_3OD, with Me_4Si as the internal standard. ESI high-resolution mass spectra (HRMS) analysis was recorded on an Autospec Ultima-TOF mass spectrometer (Micromass UK Ltd., Manchester, UK). Flash chromatography was performed on Combiflash Rf 200 (Teledyne, Nebraska, USA), particle size 0.038 mm. All test compounds were confirmed to be ≥95% pure by HPLC.

Oxysophocarpine (4). H_2O_2 (30%, 2 mL, 19.6 mmol) was added into **3** (1.0 g, 4.07 mmol) with stirring. The reaction mixture was stirred at 50 °C for 16 h and then extracted with CH_2Cl_2 to remove the unreacted **3** and concentrated under reduced pressure. The residue was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid **4** (0.7 g, 66%); mp 207−208 °C. ^{1}H NMR (CD_3OD, 400 Hz): δ 6.61−6.66 (m, 1H), 5.80 (dd, J = 9.6, 2.4 Hz, 1H), 4.48−4.55 (m, 1H), 3.87−3.92 (m, 1H), 3.74 (t, J = 12.8 Hz, 1H), 3.32−3.41 (m, 3H), 3.16−3.25 (m, 1H), 2.67−2.74 (m, 1H), 2.31−2.51 (m, 3H), 1.94−2.10 (m, 3H), 1.67−1.87 (m, 4H), 1.56−1.64 (m, 2H). HRMS: calcd for $C_{15}H_{23}N_2O_2$ $(M + H)^+$, 263.1760; found, 263.1758.

13-Hydroxymatrine (5). To a solution of KOH (33.6 g, 0.6 mol) in water (300 mL) was added **1** (12.3 g, 0.05 mol) with stirring at room temperature. The reaction mixture was refluxed for 8 h, then cooled down to 0 °C and neutralized with 3N HCl. After concentrated in vacuo, MeOH (150 mL) was added, KCl was removed in filtration, and the filtrate was concentrated under reduced pressure. The residue was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid **5** (6.2 g, 47%); mp 211−213 °C. ^{1}H NMR (CD_3OD, 400 Hz): δ 4.51 (dd, J = 4.4, 13.8 Hz, 1H), 4.14−4.18 (m, 1H), 3.86−3.92 (m, 1H), 3.47 (s, 1H), 3.36 (m, 2H), 3.24−3.26 (m, 1H), 2.89−3.04 (m, 3H), 2.52 (dd, J = 3.6, 17.6 Hz, 1H), 2.37 (dt, J = 17.6, 3.2 Hz, 1H), 2.17−2.24 (m, 1H), 1.95−2.04 (m, 2H), 1.81−1.94 (m, 3H), 1.69−1.79 (m, 4H), 1.59−1.68 (m, 1H). HRMS: calcd for $C_{15}H_{25}N_2O_2$ $(M + H)^+$, 265.1916; found, 265.1921.

General Procedures for the Synthesis of Compounds (6a−c). To a solution of **5** (0.8 g, 3 mmol) in 50% KOH aqueous (2 mL) was added dropwise halogenated hydrocarbon (3.3 mmol) with stirring. The mixture was stirred at room temperature for 8 h. After completion of the reaction, the resulting mixture was neutralized with 3N HCl and extracted with CH_2Cl_2. Combined organic phases were washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified with flash column chromatography on silica gel using EtOAc and cyclohexane as eluent to give white solid.

13-Methoxymatrine (6a). The title compound was prepared from **5** and methyl iodide in the same manner as described above (22%); mp 62−64 °C. ^{1}H NMR ($CDCl_3$, 400 Hz): δ 4.30 (dd, J = 4.4, 12.4 Hz, 1H), 3.91−3.97 (m, 1H), 3.63 (d, J = 4 Hz, 1H), 3.28 (s, 3H), 3.06 (t, J = 12.8 Hz, 1H), 2.73−2.81 (m, 2H), 2.40−2.56 (m, 2H), 2.15−2.20 (m, 1H), 2.06 (s, 1H), 1.84−1.96 (m, 3H), 1.61−1.74 (m, 4H), 1.33−1.57 (m, 6H). HRMS: calcd for $C_{16}H_{27}N_2O_2$ $(M + H)^+$, 279.2073; found, 279.2085.

13-Ethoxymatrine (6b). The title compound was prepared from **5** and ethyl iodide in the manner as described above (20%); mp 71−73 °C . ^{1}H NMR ($CDCl_3$, 400 Hz): δ 4.54−4.61 (m, 1H), 3.47−3.90 (m, 7H), 3.17 (s, 1H), 2.51−2.74 (m,

3H), 2.30−2.48 (m, 3H), 1.61−2.10 (m, 10H), 1.22 (t, J = 6.8 Hz, 3H). HRMS: calcd for $C_{17}H_{29}N_2O_2$ (M + H)$^+$, 293.2229; found, 293.2230.

13-Benzyloxymatrine (6c). The title compound was prepared from **3** and benzyl bromide in the manner as described above (57%); mp 121−123 °C. ^{1}H NMR (CDCl$_3$, 400 Hz): δ 7.24−7.34 (m, 5H), 4.47−4.56 (m, 2H), 4.35 (dd, J = 4.0, 12.8 Hz, 1H), 3.99−4.05 (m, 1H), 3.84 (d, J = 4.0 Hz, 1H), 3.10 (t, J = 12.8 Hz, 1H), 2.76−2.84 (m, 2H), 2.45−2.65 (m, 2H), 2.16−2.23 (m, 1H), 2.09 (s, 1H), 1.82−1.98 (m, 3H), 1.33−1.77 (m, 10H). HRMS: calcd for $C_{22}H_{31}N_2O_2$ (M + H)$^+$, 355.2386; found, 355.2371.

13-Formyloxymatrine (6d). To a solution of **5** (1.0 g, 3.79 mmol) in anhydrous CH_2Cl_2 (10.0 mL) was added dropwise benzoyl choride (0.48 mL, 4.17 mmol) with stirring, and then KOH (0.42 g, 7.5 mmol) powder was added in portion. The mixture was stirred at room temperature for 4 h. Inorganic salt was removed via filtration, and the filtrate was concentrated under reduced pressure. The residue was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid (0.51 g, 37%); mp 108−110 °C. ^{1}H NMR (CDCl$_3$, 400 Hz): δ 7.96 (d, J = 7.2 Hz, 2H), 7.56 (t, J = 7.6 Hz, 1H), 7.41−7.45 (m, 2H), 5.38−5.39 (m, 1H), 4.32 (dd, J = 4.4, 12.8 Hz, 1H), 4.05 (m, 1H), 3.10 (t, J = 12.8 Hz, 1H), 2.47−2.81 (m, 4H), 2.40−2.46 (m, 1H), 2.17 (s, 1H), 1.91−2.03 (m, 3H), 1.39−1.82 (m, 10H). HRMS: calcd for $C_{22}H_{29}N_2O_3$ (M + H)$^+$, 369.2178; found, 369.2188.

13-Nitromethylmatrine (7a). A solution of nitromethane (1.08 mL, 20 mmol) and **3** (4.92 g, 20 mmol) in acetonitrile (10 mL) was mixed with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 3.04 g, 20 mmol) at room temperature. The resulting solution was kept at room temperature for 24 h and poured into water (50 mL). The mixture was acidified with diluted hydrochloric acid (pH 2) and then extracted with CH_2Cl_2. The combined CH_2Cl_2 extracts were washed with water, dried over Na_2SO_4, and evaporated in vacuo. The residue was purified with flash column chromatography on silica gel using EtOAc and MeOH as eluent to give white solid (2.90 g, 47%); mp 80−82 °C. ^{1}H NMR (CDCl$_3$, 400 Hz): δ 4.28−4.39 (m, 3H), 4.04−4.09 (m, 1H), 3.12 (t, J = 12.4 Hz, 1H), 2.78−2.85 (m, 3H), 2.56 (dd, J = 5.2, 17.2 Hz, 1H), 2.16−2.24 (m, 2H), 1.91−2.01 (m, 5H), 1.82−1.90 (m, 3H), 1.51−1.78 (m, 3H), 1.38−1.48 (m, 3H). HRMS: calcd for $C_{16}H_{26}N_3O_3$ (M + H)$^+$, 308.1974; found, 308.1973.

13-Methylaminomatrine (7b). Na (0.46 g, 20 mmol) was added to methylamine in EtOH (60 mL) in portion with stirring at 0 °C; after the completion of the reaction, **3** was added (2.46 g, 10 mmol) at the same temperature. The reaction mixture was stirred for 24 h at room temperature and concentrated in vacuo, then soaked in ether. The mixture was filtered, and the solid was washed with ether; the filtrate was concentrated under reduced pressure to remove ether. The residue was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid **5b** (1.1 g, 40%); mp 80−81 °C. ^{1}H NMR (CDCl$_3$, 400 Hz): δ 4.34 (dd, J = 4.4, 12.8 Hz, 1H), 4.00 (br, 1H), 3.10 (t, J = 12.8 Hz, 1H), 2.94 (m, 1H), 2.83 (m, 2H), 2.56 (dd, J = 4.4, 16.8 Hz, 1H), 2.46 (s, 3H), 2.32 (dd, J = 5.6, 16.8 Hz, 1H), 2.12 (s, 1H), 1.89−1.97 (m, 4H), 1.63−1.77 (m, 5H), 1.37−1.54 (m, 6H). HRMS: calcd for $C_{16}H_{28}N_3O$ (M + H)$^+$, 278.2232; found, 278.2243.

13,14-Dihydroxymatrine (8). To a solution of **3** (6.15 g, 25 mmol) in water (20 mL) and acetone (20 mL) was added $KMnO_4$ (5.93 g, 37.5 mmol) in portion over 1 h with stirring at 0 °C. After confirming the completion of the reaction in TLC, the mixture was stirred at 0 °C for another 1 h and then MeOH (50 mL) was added. The mixture was filtered, and the solid was washed with MeOH, and the filtrate was concentrated under reduced pressure to remove MeOH and acetone. The resulting solution was added 10% NaOH aqueous to adjust pH to 10−11, extracted with CH_2Cl_2, dried over Na_2SO_4, and concentrated in vacuo, soaked in ether, filtered, and dried to give white solid **6** (3.3 g, 47%); mp 156−158 °C. ^{1}H NMR (CD$_3$Cl, 400 Hz): δ 4.27 (s, 1H), 4.09−4.18 (m, 3H), 3.96 (s, 1H), 3.21 (t, J = 12.4 Hz, 1H), 2.80 (m, 2H), 2.60 (s, 1H), 2.38 (d, J = 13.2 Hz, 1H), 2.07 (m, 1H), 1.85−2.04 (m, 3H), 1.41−1.71 (m, 10H). HRMS: calcd for $C_{15}H_{25}N_2O_3$ (M + H)$^+$, 281.1865; found, 281.1882.

General Procedures for the Synthesis of Compounds 9a−b. To a solution of **8** (3 mmol) in 50% KOH aqueous (2 mL) was added dropwise halogenated hydrocarbon (6.5 mmol) with stirring. The mixture was stirred at room temperature for 8 h. After completion of the reaction, the resulting mixture was neutralized with 3N HCl and then extracted with CH_2Cl_2. The combined organic phase was washed with water and brine, dried over Na_2SO_4, and then concentrated in vacuo. The residue was purified with flash column chromatography on silica gel using CH_2Cl_2 as eluent to give white solid.

13,14-Dimethoxymatrine (9a). The title compound was prepared from **8** and methyl iodide in the same manner as described above (35%); mp 77−79 °C. ^{1}H NMR (CD$_3$OD, 400 Hz): δ 4.16 (dd, J = 4.4, 12.8 Hz, 1H), 3.78−3.84 (m, 1H), 3.60−3.63 (m, 1H), 3.56−3.57 (m, 1H), 3.44 (s, 3H), 3.35 (s, 3H), 3.02 (t, J = 12.8 Hz, 1H), 2.74−2.81 (m, 2H), 2.14−2.20 (m, 2H), 1.91−2.02 (m, 3H), 1.21−1.76 (m, 10H). HRMS: calcd for $C_{17}H_{29}N_2O_3$ (M + H)$^+$, 309.2178; found, 309.2191.

13,14-Dibenzyloxymatrine (9b). The title compound was prepared from **6** and benzyl bromide in the same manner as described above (81%); mp 105−108 °C. ^{1}H NMR (CD$_3$OD, 400 Hz): δ 7.21−7.43 (m, 10H), 4.99 (d, J = 12.4 Hz, 1H), 4.66 (m, 3H), 4.45 (m, 2H), 3.30−3.90 (m, 6H), 2.69 (m, 2H), 2.07−2.27 (m, 3H), 1.59−1.89 (m, 8H), 1.39 (m, 1H). HRMS: calcd for $C_{29}H_{37}N_2O_3$ (M + H)$^+$, 461.2804; found, 461.2820.

General Procedures for the Synthesis of Compounds (9c−j). To a solution of **8** (3 mmol) in anhydrous CH_2Cl_2 (5.0 mL) was added dropwise corresponding acyl chloride (9 mmol) with stirring. The mixture was stirred at room temperature for 1−24 h and then concentrated in vacuo to give crude products.

13,14-Diethoxyacyloxymatrine (9c). The title compound was prepared from **8** and ethyl chloroformate in the same manner as described above in the presence of KOH powder, stirred 8 h, and the crude product purified with flash column chromatography on silica gel using CH_2Cl_2 as eluent to give white solid (30%); mp 64−66 °C. ^{1}H NMR (CD$_3$OD, 400 Hz): δ 5.31 (d, J = 2.8 Hz, 1H), 5.23 (d, J = 2.8 Hz, 1H), 4.10−4.20 (m, 4H), 3.86−3.93 (m, 1H), 3.09 (t, J = 12.8 Hz, 1H), 2.73−2.80 (m, 2H), 2.46 (dt, J = 15.2, 5.6 Hz, 1H), 2.14 (s, 1H), 1.93−2.01 (m, 2H), 1.72−1.88 (m, 2H), 1.39−1.71 (m, 10H), 1.24 (m, 6H). HRMS: calcd for $C_{21}H_{33}N_2O_7$ (M + H)$^+$, 425.2282; found, 425.2325.

13,14-Di(4-fluoro-3-nitro)benzoyloxymatrine (9d). The title compound was prepared from **8**, 4-fluoro-3-nitro-1-benzenecarbonyl chloride, and K_2CO_3 anhydrous in the same manner as described above, stirred 24 h, and the crude product was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid (31%); mp 115−117 °C. ^{1}H NMR (CD$_3$OD, 400 Hz): δ 8.62 (dd, J = 7.2, 2.4 Hz, 1H), 8.48 (dd, J = 7.2, 2.0 Hz, 1H), 8.34−8.38 (m, 1H), 8.22−8.26 (m, 1H), 7.54−7.59 (m, 1H), 7.45−7.50 (m, 1H), 5.83−5.87 (m, 2H), 4.28 (dd, J = 13.2, 4.4 Hz, 1H), 4.09−4.16 (m, 1H), 3.23 (t, J = 12.8 Hz, 1H), 2.80−2.84 (m, 2H), 2.61−2.67 (m, 1H), 2.22 (s, 1H), 1.41−2.12 (m, 13H). HRMS: calcd for $C_{29}H_{29}F_2N_4O_9$ (M + H)$^+$, 615.1903; found, 615.1885.

14-Hydroxy-13-acetoxymatrine Hydrochloride (9e). The title compound was prepared from **8** and acetyl chloride in the same manner as described above, stirred 8 h, and the crude product purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid (21%); mp 194−196 °C. ^{1}H NMR (CDCl$_3$, 400 Hz): δ 12.22 (s, 1H), 5.19 (s, 1H), 5.00 (s, 1H), 4.63 (m, 1H), 4.56 (dd, J = 4, 14.4 Hz, 1H), 4.33−4.40 (m, 2H), 3.74 (t, J = 13.6 Hz, 1H), 3.51−3.59 (m, 2H), 3.13 (d, J = 9.2 Hz, 1H), 2.52−2.66 (m, 3H), 2.33−2.43 (m, 2H), 2.02−2.13 (m, 3H), 1.87−1.90 (m, 1H), 1.63−1.75 (m, 6H). HRMS: calcd for $C_{17}H_{27}N_2O_4$·HCl (M − Cl)$^+$, 323.1971; found, 323.1966.

14-Hydroxy-13-chloroacetoxymatrine Hydrochloride (9f). The title compound was prepared from **8** and chloroacetyl chloride in the same manner as described above, stirred 5 h, and the crude product purified with flash column chromatography on silica gel using CH_2Cl_2 and EtOH as eluent to give white solid (25%); mp 226−227 °C . ^{1}H NMR (CD_3OD, 400 Hz): δ 5.43 (s, 1H), 4.41−4.42 (m, 1H), 4.32 (d, J = 15.2 Hz, 1H), 4.19 (d, J = 15.2 Hz, 2H), 3.95 (m, 1H), 3.36−3.57 (m, 4H), 2.97−3.05 (m, 3H), 2.46−2.50 (m, 1H), 1.69−2.07 (m, 10H). HRMS: calcd for $C_{17}H_{26}ClN_2O_4 \cdot HCl$ (M − Cl)$^+$, 357.1581; found, 357.1582.

14-Hydroxy-13-(2-chloro)propionyloxymatrine Hydrochloride (9g). The title compound was prepared from **8** and 2-chloropropionyl chloride in the same manner as described above, stirred 5 h, and the crude product purified with recrystallization with EtOH twice to give white solid (51%); mp 239−240 °C. ^{1}H NMR (CD_3OD, 400 Hz): δ 5.32−5.34 (m, 1H), 4.58−4.63 (m, 1H), 4.37 (dt, J = 4.4, 14 Hz, 1H), 4.26 (d, J = 2.8 Hz, 1H), 3.77−3.84 (m, 1H), 3.50 (s, 1H), 3.37 (t, J = 13.2 Hz, 2H), 3.02 (m, 2H), 2.87 (t, J = 13.6 Hz, 1H), 2.36 (dt, J = 5.2, 14.4 Hz, 1H), 1.96−2.10 (m, 3H), 1.71−1.86 (m, 8H), 1.67−1.69 (m, 3H). HRMS: calcd for $C_{18}H_{28}ClN_2O_4 \cdot HCl$ (M − Cl)$^+$, 371.1738; found, 371.1753.

14-Hydroxy-13-benzoyloxymatrine Hydrochloride (9h). The title compound was prepared from **8** and benzoyl chloride in the same manner as described, stirred 3 h, and the crude product was purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid (31%); mp 225−226 °C. ^{1}H NMR (CD_3OD, 400 Hz): δ 8.10 (d, J = 7.6 Hz, 2H), 7.58 (t, J = 7.6 Hz, 1H), 7.44 (t, J = 7.6 Hz, 2H), 5.55 (s, 1H), 4.37−4.44 (m, 2H), 3.79−3.86 (m, 1H), 3.51 (s, 1H), 3.01−3.06 (m, 2H), 2.86 (t, J = 13.6 Hz, 1H), 2.38−2.44 (m, 1H), 1.79−2.13 (m, 12H), 1.12 (t, J = 6.8 Hz, 1H). HRMS: calcd for $C_{22}H_{29}N_2O_4 \cdot HCl$ (M − Cl)$^+$, 385.2127; found, 385.2131.

14-Hydroxy-13-(4-fluoro-3-nitro)benzoyloxymatrine Hydrochloride (9i). The title compound was prepared from **8** and 4-fluoro-3-nitro-1-benzenecarbonyl chloride in the same manner as described above, stirred 1 h, and the crude product purified with flash column chromatography on silica gel using CH_2Cl_2 and MeOH as eluent to give white solid (31%); mp 103−105 °C. ^{1}H NMR (CD_3OD, 400 Hz): δ 8.60 (dd, J = 2.0, 7.2 Hz, 1H), 8.26−8.30 (m, 1H), 7.49−7.53 (m, 1H), 5.60 (t, J = 2.8 Hz, 1H), 4.24−4.30 (m, 2H), 3.88−3.95 (m, 1H), 3.14 (t, J = 12.8 Hz, 1H), 2.79 (d, J = 11.2 Hz, 2H), 2.53 (dt, J = 5.6, 14.8 Hz, 1H), 2.24 (s, 1H), 2.01−2.06 (t, J = 10.8 Hz, 2H), 1.38−1.89 (m, 11H). HRMS: calcd for $C_{22}H_{27}FN_3O_6 \cdot HCl$ (M − Cl)$^+$, 448.1883; found, 448.1881.

14-Hydroxy-13-ethoxyacyloxymatrine (9j). The title compound was prepared from **8**, ethyl chloroformate, and K_2CO_3 anhydrous as described above, stirred 12 h, and the crude product purified with flash column chromatography on silica gel using CH_2Cl_2 and EtOH as eluent to give white solid (28%); mp 50−52 °C. ^{1}H NMR ($CDCl_3$, 400 Hz): δ 4.99−5.03 (m, 1H), 4.17−4.29 (m, 4H), 3.96−4.00 (m, 1H), 3.13 (t, J = 12.8 Hz, 1H), 2.76−2.83 (m, 2H), 2.17−2.22 (m, 1H), 2.09−2.13 (m, 2H), 1.91−1.99 (m, 2H), 1.34−1.84 (m, 10H), 1.28−1.32 (m, 4H). HRMS: calcd for $C_{18}H_{29}N_2O_5$ (M + H)$^+$, 353.2077; found, 353.2076.

14-Methoxymatridin-15-one (10). To a solution of **6** (0.84 g, 3 mmol) in 50% KOH aqueous (2 mL) was added dropwise methyl iodide (0.37 mL, 6 mmol) with stirring, and then acetone (1 mL) was added. The mixture was stirred at room temperature for 8 h. After completion of the reaction, the resulting mixture was neutralized with 3N HCl and then extracted with CH_2Cl_2. The combined organic phase was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified with flash column chromatography on silica gel using CH_2Cl_2 as eluent to give white solid **10** (0.30 g, 36%); mp 110−111 °C. ^{1}H NMR (CD_3OD, 400 Hz): δ 5.43 (t, J = 4.0 Hz, 1H), 3.99 (dd, J = 4.4, 13.0 Hz, 1H), 3.80−3.86 (m, 1H), 3.53 (s, 3H), 3.12 (t, J = 12.8 Hz, 1H), 2.77 (t, J = 10.8 Hz, 2H), 2.65 (dt, J = 6, 17.6 Hz, 1H), 2.14−2.24 (m, 2H), 1.84−2.00 (m, 3H), 1.54−1.76 (m, 6H), 1.40−1.49 (m, 3H). HRMS: calcd for $C_{16}H_{25}N_2O_2$ (M + H)$^+$, 277.1916; found, 277.1908.

Biological Methods. Cell Lines. Human HepG2 liver cells were from the American Tissue Culture Collection (ATCC, Fredrick, MD). HepG2.2.15 cells were used for anti-HBV test. The cells were cultivated in basic MEM supplemented with 10% of fetal bovine serum. Cells were digested with 0.05% Trypsin-EDTA and split twice a week. Huh7.5 human liver cells (kindly provided by Vertex Pharmaceuticals Inc., Boston, MA) were culture in the Dulbecco's Minimal Essential Medium supplemented with 10% inactivated fetal bovine serum and 1% penicillin−streptomycin. Cells were digested with 0.05% Trypsin−EDTA and split twice a week.

Hsc70 mRNA Expression. HepG2.2.15 cells were planted into 24-well plate with a density of 5×10^4 per well in a complete medium (Opti-MEM, 10% FBS, Invitrogen), incubated for one day, and then treated with the study compounds (100 μg/mL) for 24 h. Total cellular RNAs were isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA) according to the instructions from the venders. The Hsc70 mRNA level was analyzed with real-time RT-PCR using SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) in the BIO-RAD iQ5Multicolor real-time detection system (Bio-Rad, Hercules, CA). The gene copy numbers were normalized to that of GAPDH. The Hsc70 and GAPDH mRNA were tested using human primers (Hsc70 primers: forward, 5′-tgctgctgctattgcttacg-3′; reverse, 5′-tcaatagtgaggattgacacatca-3′; GAPDH primers: forward, 5′-acccactcctccacctttg-3′; reverse, 5′-ctgtagccaaattcgtt gtcat-3′). The data were analyzed with Bio-Rad iQ5 software.

Anti-HBV Effect in Vitro. HepG2.2.15 cells at 2×10^4 cells per well (96-well microplates, Falcon, Oxnard, CA, USA) were treated with compounds with nontoxic doses at 37 °C for 9 days, respectively. Cellular DNA was extracted with a commercial kit (Viral genome DNA Mini extraction kit, BioDev). Cells HBV DNA level was quantified with real time PCR.

For Southern blot, DNA samples were subjected to denaturation in a solution containing 0.5 M NaOH and 1.5 M NaCl, followed by neutralization in a buffer containing 1 M Tris-HCl (pH 7.4) and 1.5 M NaCl. DNA was then blotted onto a Hybond-C membrane (GE Healthcare) in 20× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). For the detection of HBV DNA, membranes were probed with an [α-^{32}P]CTP (Perkin-Elmer)-labeled HBV probe. Hybridization was carried out in 10 mL hybridization buffer with 4 h prehybridization and overnight hybridization at 42 °C, followed by 20 min wash with 0.1× SSC and 0.1% SDS once at 65 °C and twice at room temperature. The membrane was exposed to the Kodak film at −70 °C for 72 h; the hybridization signals were quantified by absolute integrated optical density (IOD) scanning using Gel-Analyzer version 3.0 software (Media Cybernetics, Silver Spring, MD, USA).

DHBV DNA Polymerase Activity. The DHBV replicative complexes were isolated from DHBV-infected duck liver by sucrose gradient centrifugation according to Offensperger WB.[15] Endogenous polymerase activity was assayed under optimal conditions in 20 μL of polymerase buffer containing 50 mM Tris−HCl (pH 8.0), 200 mM KCl, 183 mM $MgCl_2$, 0.3% β-mercaptoethanol, 0.5% Nonidet P-40, 100 mM each of dATP, dGTP, dCTP, and 0.2 μM ^{3}H-dTTP. The reactions with a final volume of 60 μL were initiated by addition of 20 μL of core preparation (replicative complexes), and 20 μL of study compounds at a concentration of 0 (as control) or 1000 μg/mL into the polymerase buffer. PFA was used as a positive control. After 90 min incubation at 37 °C, the reactions were terminated by dispensing the reaction mixture onto filter paper. The filter paper was washed three times with buffer containing 5% trichloroacetic acid and once with absolute ethanol, dried, and

counted in a scintillation counter (LS-6000, Beckman Coulter, USA).

Anti-HBV Effect on Lamivudine-Resistant HBV Strain. Huh-7.5 cells per well (2×10^5) were plated into 12-well plates. After overnight incubation, the cells were transiently transfected respectively with pcDNA3.1 vectors containing wild-type HBV strain or lamivudine-resistant strain (rtL 180M + reM204V) for 24 h. The supernatant was discarded, followed by PBS wash. Then, the cells were cultivated with fresh medium containing lamivudine (0.16 μg/mL) or **6b** (37 μg/mL). After 36 h treatment, HBV DNA replicative intermediate was extracted using method reported before,[15] and the level was quantified using real time PCR.

Safety Evaluation in Vivo. Male and female ICR mice with weight of 22.0 $\pm$ 1.0 g were purchased from the Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). They were fed with regular rodent chow and housed in an air-conditioned room. The mice were randomly divided into four groups with 10 mice each (5 male plus 5 female). The four groups of mice orally received **6b** at 0 (1% polysorbate-80 in saline as control), 250, 500, or 750 mg/kg, respectively. **6b** was given in a single-dosing. Body weight as well as survival was monitored. Seven days later, blood samples were taken for liver and kidney function examination.

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Supporting Information Available: HPLC analytical data for final compounds are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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