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5-Iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones as non-nucleoside anti-HBV agents

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A series of 5-iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones, which can be considered as S-DABO derivatives, have been synthesized and their antiviral effect on extracellular HBV DNA was evaluated using the HepAD38 cell system. Compounds **6d1** and **6e3** exhibited more potent anti-HBV activity than lamivudine with EC_{50} values of 0.376μM and 0.469 μM, respectively. In addition, inhibition of intracellular HBV DNA, pgRNA, HBeAg and HBsAg of compounds **6d1** and **6e3** were detected to initially infer the action mechanism. RT-PCR analysis of pgRNA demonstrated that these new S-DABOs analogues could not interfere with HBV transcription. TRFIA analysis revealed that compounds **6d1** and **6e3** effectively reduced the secretion of HBeAg. These results demonstrated that 5-iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones possess anti-HBV ability and could be used as potential agents against HBV infection with an additional merit of low cytotoxicity.

1.Introduction

The hepatitis B virus (HBV) belongs to Hepadnaviridae, and its infection is a major cause of viral hepatitis worldwide.[1] Acute HBV infection occasionally results in fulminant hepatitis and usually progresses to a chronic state, which could lead to liver cirrhosis and hepatocellular carcinoma (HCC).[2,3] Approximately 350 million people worldwide are chronically infected with this virus, which results in 0.5 -1.2 million deaths annually.

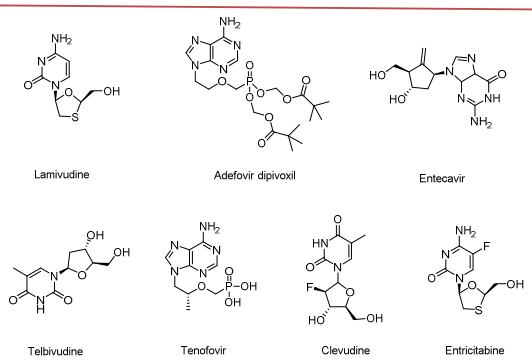

Figure 1. Clinical used anti-HBV drugs

Currently, interferon α and nucleoside analogues are widely used in controlling the progression of chronic hepatitis B. However, interferon α is less effective in curing HBV infection and has some adverse effects.[4] Nucleoside analogues (Figure 1) could selectively inhibit the viral polymerase, but the required long-term therapy might lead almost invariably to resistance problems.[5] Under these circumstances, it is urgent to find effective anti-HBV agents as nonnucleosides with different action mechanism.

So far, the clinical used anti-HBV drugs are nucleoside analogues (Figure 1), which need to be transformed to their active triphosphate forms by cellular kinases. As chain-terminators, the triphosphate nucleoside inhibitors compete with natural nucleoside substrates for incorporation into the elongating DNA chain.[6]

Interestingly, most of these nucleoside analogues are also efficient against human immunodeficiency virus(HIV). Lamivudine[7], Tenofovir[7] and Entricitabine[8] have been clinically approved for the treatment of HIV infection. In addition, Adefovir dipivoxil[9,10], Entecavir[11] and Telbivudine[12] are also reported with anti-HIV activity.

As reported in literature, the polymerase of HBV is structurally related with the reverse transcriptase of HIV-1[13]. Therefor, some compounds with anti-HIV activity were screened for anti-HBV. Lee and his co-workers successfully developed 2,5-pyridinedicarboxylic acid derivatives, which originated from non-nucleoside HIV-1 RT inhibitors[14], as anti agent of HBV.

As anti-HIV-1 non-nucleoside reverse transcriptase inhibitors(NNRTIs), dihydroalkylthio benzyloxopyrimidines(S-DABO) derivatives have attracted attention due to their high specificity, excellent potency and low cytotoxicity.[15]

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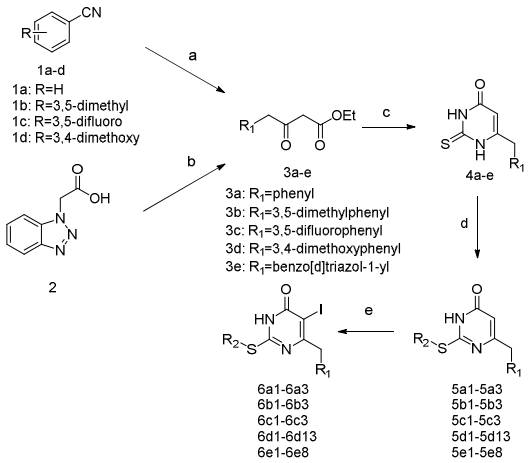

Reagents and conditions: (a) ethyl bromoacetate, Zn/ THF, reflux. (b) $MgCl_2$, Et_3N, CDI, CH_3CN, $CH_2(COOEt)(COOK)$, rt. 12h and then reflux 2h (c) thiourea, EtONa/EtOH, reflux. (d) substituted alkyl halide, K_2CO_3, rt. (e) NIS, DMF, 0°C.

Scheme 1. Synthesis of target compounds **6**

However, little attention has been paid to their anti-HBV activity. According to the SAR of S-DABO derivatives as HIV-1 RT inhibitors, both substituents on the side chain of C-2 and C-6 were important for antiviral activity. Especially, the iodine atom at the 5-position would provide a halogen bond which could be beneficial for the inhibitory activity.[16] To widen the antiviral spectrum of S-DABOs, we decided to explore the different structural modifications of the S-DABOs, including structural diversity at both the side chain on C-2 and C-6 position with bearing a iodine atom at the 5-position. Firstly, we screened the activity of target compounds against HBV on extracellular HBV DNA. For the active compounds, their inhibition of intracellular HBV DNA, pgRNA, HBeAg and HBsAg were detected to initially infer the action mechanism.

Results and discussion

2.1 Chemistry

A general synthesis of target compounds is outlined in Scheme 1. The β-ketoesters **3a-d** were obtained by reaction of substituted benzyl cyanide with zinc and ethyl 2-bromoacetate using the method of Hannick and Kishi.[17] However, compounds **3e** was prepared with an alternative method reported by Clay et al.[18] through the reaction of benzo[d]triazol-1-yl acetic acid **2** with *N,N'*-carbonyldiimidazole (CDI) followed by treatment with ethyl potassium malonates in the presence of anhydrous $MgCl_2$ and Et_3N. Condensation of **3a-e** with thiourea in the presence of sodium ethoxide in boiling ethanol provided the 6-aryl thiouracil **4a-e**. The S-DABO analogues **5a-e** were prepared from S-alkylation with appropriate substituted alkyl halide using potassium carbonate in anhydrous DMF or sodium ethoxide in ethanol[15,19,20]. Subsequent treatment of **5a-e** with NIS in DMF at 0°C afforded the desirable target compounds **6a-e**[21]. The structure assignments of these compounds were identified by NMR and mass spectral data.

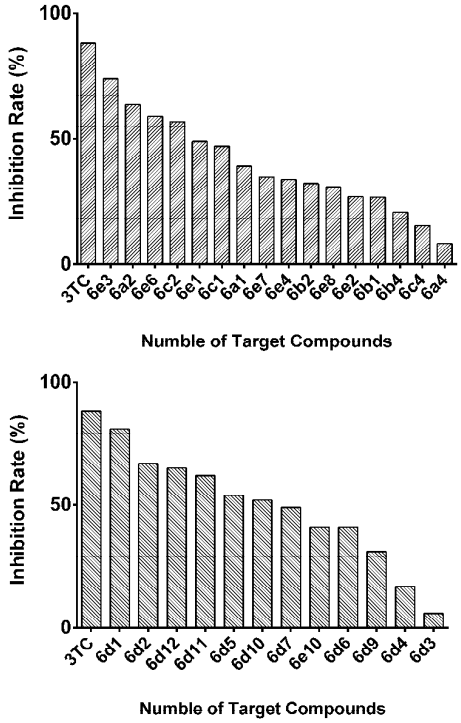

Figure 2. Preliminary viral screening on extracellular HBV DNA in HepAD38 cells

2.2 Results and discussion

To preliminary screen the antiviral activity of compounds on extracellular HBV DNA, all the target compounds were treated with HepAD38 cells at one concentration(10μM) and lamivudine was used as a reference antiviral drug (Figure 2). The cell supernatants were collected and analyzed by qRT-PCR (Quantitative Real-time PCR). The results are summarized in Table 1.

As shown in Table 1, most of the target compounds displayed moderate activity against HBV replication. Especially, **6d1** (81%) displayed potent HBV inhibitory activity close to lamivudine (88%).

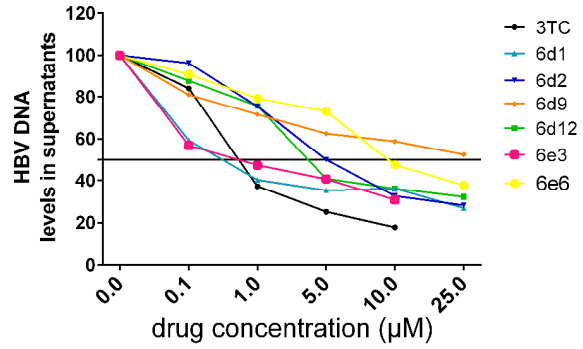

Figure 3. Dose-dependent effect on extracellular HBV DNA of six candidate compounds in HepAD38 cells

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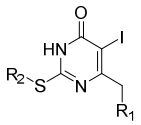
Table 1. Inhibition of extracellular HBV DNA by target compounds



compd	R_1	R_2	inhibition[ab]	compd	R_1	R_2	inhibition
6a1	phenyl	(p-NO_2)$C_6H_5CH_2$	NA[c]	6d1	3,4-dimethoxyphenyl	(p-NO_2)$C_6H_5CH_2$	81%
6a2	phenyl	(p-CN)$C_6H_5CH_2$	63%	6d2	3,4-dimethoxyphenyl	(p-CN)$C_6H_5CH_2$	67%
6a3	phenyl	(p-NO_2)$C_6H_5OCH_2CH_2$	13%	6d3	3,4-dimethoxyphenyl	(p-NO_2)$C_6H_5OCH_2CH_2$	6%
				6d4	3,4-dimethoxyphenyl	(p-I)$C_6H_5CH_2$	17%
6b1	3,5-dimethylphenyl	(p-NO_2)$C_6H_5CH_2$	25%	6d5	3,4-dimethoxyphenyl	(p-CF_3)$C_6H_5CH_2$	54%
6b2	3,5-dimethylphenyl	(p-CN)$C_6H_5CH_2$	33%	6d6	3,4-dimethoxyphenyl	3,5-Dimethoxybenzyl	41%
6b3	3,5-dimethylphenyl	(p-NO_2)$C_6H_5OCH_2CH_2$	20%	6d7	3,4-dimethoxyphenyl	(p-CH_3)$C_6H_5OCH_2CH_2$	49%
				6d8	3,4-dimethoxyphenyl	(p-Br)$C_6H_5OCH_2CH_2$	NA
6c1	3,5-difluorophenyl	(p-NO_2)$C_6H_5CH_2$	47%	6d9	3,4-dimethoxyphenyl	cycloheptyl	31%
6c2	3,5-difluorophenyl	(p-CN)$C_6H_5CH_2$	14%	6d10	3,4-dimethoxyphenyl	cyclohex-2-en-1-yl	52%
6c3	3,5-difluorophenyl	(p-NO_2)$C_6H_5OCH_2CH_2$	9%	6d11	3,4-dimethoxyphenyl	(o-NO_2)$C_6H_5CH_2$	62%
				6d12	3,4-dimethoxyphenyl	(m-Cl)$C_6H_5CH_2$	65%
6e1	benzo[d]triazol-1-yl	(p-NO_2)$C_6H_5CH_2$	49%	6d13	3,4-dimethoxyphenyl	(m-CH_3O)$C_6H_5CH_2$	NA
6e2	benzo[d]triazol-1-yl	(p-CN)$C_6H_5CH_2$	27%				
6e3	benzo[d]triazol-1-yl	(p-NO_2)$C_6H_5OCH_2CH_2$	74%	6e6	benzo[d]triazol-1-yl	3,5-Dimethoxybenzyl	59%
6e4	benzo[d]triazol-1-yl	(p-I)$C_6H_5CH_2$	34%	6e7	benzo[d]triazol-1-yl	(p-CH_3)$C_6H_5OCH_2CH_2$	35%
6e5	benzo[d]triazol-1-yl	(p-CF_3)$C_6H_5CH_2$	NA	6e8	benzo[d]triazol-1-yl	(p-Br)$C_6H_5OCH_2CH_2$	31%
3TC			88%				

[a]The data are expressed as percent inhibition of viral DNA in the presence of 10μM of the test compounds as compared to untreated infected controls. [b]Data represent mean values of at least two experiments. [c]NA=not active at 10μM

Considering the different substitutions at C-2 position for compounds **6d**, electron withdrawing groups, particularly nitro (**6d1**,81%) and cyano (**6d2**,67%) would be beneficial for the inhibitory activity. For **6e** subseries, bearing benzotriazole on the C-6 position, compound **6e3** (74%) represented the most active derivative.

After the preliminary screen at one concentration, six compounds with high inhibition rates were selected to perform in dose response assays (Figure 3) using the same HepAD38 cells and the concentrations required to inhibit 50% of HBV DNA (EC_{50}) are summarized in Table 2. We also determined the cytotoxic effects through the MTT assay and the cytotoxicity of each compound was expressed as the concentration of compound required to cause 50% cell death (CC_{50}) of the HepAD38 cells (Table 2).

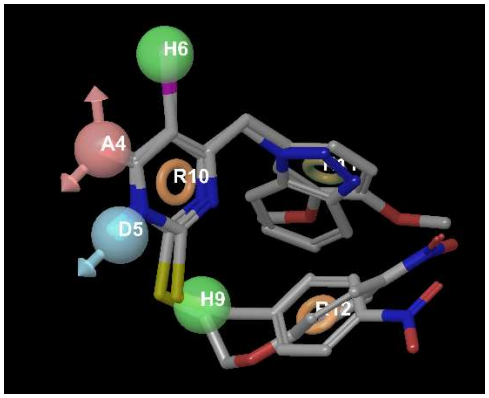
As shown in Table 2, most of the selected compounds displayed activity against HBV with low cytotoxicity. Among them, compound **6d1** and compound **6e3** showed excellent anti-HBV activity with EC_{50} values of 0.376μM and 0.469 μM,respectively, which were more potent than reference drug lamivudine(0.731 μM).

Table 2. In vitro anti-HBV activity and cytotoxicity of the target compounds

Compd	EC_{50}(μM)[a]	CC_{50}(μM)[b]	SI[c]
6d1	0.376	124	330
6d2	4.84	92	19
6d9	40.8	98	2.4
6d12	4.28	119	27.8
6e3	0.469	52	111
6e6	12.16	146	12
3TC	0.731	>100	>137

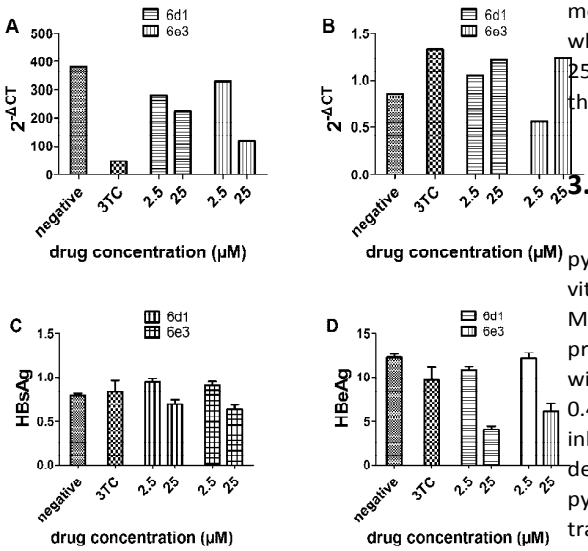
[a]Concentration inhibiting viral replication by 50%. [b]50% cytotoxicity concentration [c]Selecivity index , defined by CC_{50}/EC_{50}.

Figure 4. Pharmacophore model of the 5-iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones. A4 is a H-bond acceptor (shown as a pink ball). D5 is a H-bond donor (shown as a blue ball). H6 and H9 are hydrophobic points (shown as green balls). R10, R11, R12 are aromatic rings (shown as orange circles).



The biological results further demonstrated that some compounds with electron withdrawing groups at C-2 position, such as **6d1** and **6e3** (p-NO_2), **6d2** (p-CN), **6d12** (m-Cl), showed significant anti-HBV activity (Table 2). In addition, compound **6d9** was found to be less active when compared to lamivudine, so the introduction of aliphatic ring at the C-2 side chain would decrease the antiviral activity. Because the present study is a preliminary structural modification, the Structure-Activity Relationships (SARs) of 5-iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones remain to be further studied.

Figure 5. (A) HBV core-associated DNA in intracellular core particles; (B) The levels of pgRNA; (C) Effects on HBV viral HBsAg secretion, Data represents the mean value ± SD; (D)Effects on HBV viral HBeAg secretion, Data represents the mean value ± SD.



The designed and synthesized 5-iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones represent a new series of anti-HBV compounds. The target of our compounds is not clarified, therefore the construction of a pharmacophoremodel is a necessary method for further structural optimization.

According to the anti-HBV activities, compounds **6d1** and **6e3** were selected to generate pharmacophore models using "Develop Common Pharmacophore Hypotheses" module of Phase (Figure 4). Several pharmacophore points were identified including three aromatic rings, two hydrophobic points, one H-bond acceptor and one H-bond donor point. Three phenyl groups (R10, R11, R12) were recognized as aromatic ring. R10 is identical among the compounds. R11 highlighted the importance of the substituted phenyl group attached to the C-2 side chain, which was consistent with the SAR results. R12 was also recognized as aromatic ring, indicating that aromatic group at C-6 was beneficial for the anti-HBV activity. The iodine atom at C-5 was identified as a hydrophobic point (H6). Moreover, the hydrogen attached to the N atom was recognized as a H-bond donor point (D5). However, A4 and H9 points are common structural elements among the synthesized compounds.

The selectivity index(SI), determined as the ratio of CC_{50} value to EC_{50} value, was an important pharmaceutical parameter that estimates possible future clinical development (Table 2). Therefore, we chose two compounds (**6a1**,**6e3**) with high SI values for further study. Huh7 cell system was used to detect intracellular HBV DNA, pgRNA, HBsAg and HBeAg (Figure 5) .

As shown in Figure 5, after the treatment of two compounds on Huh7 cells, Intracellular HBV DNA analysis was performed by qRT-PCR (Figure 5A). The result showed that **6d1** and **6e3** could efficiently impede the replication of intracellular HBV DNA. RT-PCR analysis of pgRNA (Figure 5B) indicated that the addition of the two compounds did not suppress the transcription of HBV genome. Figure 5C and Figure 5D showed the secretion of HBsAg and HBeAg in treated Huh 7 cell culture media. The tested two compounds affected HBsAg slightly, whereas the effect on HBeAg secretion could be observed at 25μM. These results suggested that **6d1** and **6e3** would reduce the secretion of HBeAg in a dose-dependent manner.

3.Conclusions

In summary, a series of 5-iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones were synthesized and tested for their in vitro anti-HBV activity, using lamivudine as a reference control. Most of the compounds revealed anti-HBV activity. The most promising results were observed for compounds **6d1** and **6e3**, with EC_{50} values against HBV DNA replication of 0.376μM and 0.469μM, respectively. In addition, they have a potent inhibitory effect on HBeAg secretion. The work of pgRNA detection demonstrated that 5-iodo-2-arylalkylthio-6-aryl pyrimidin-4(3H)-ones could not interfere with HBV transcription. Further investigation of the mechanism of action are ongoing in our laboratories.

4.Experimental

4.1 Chemistry

NMR spectra were recorded on a Bruker AVANCE Ⅲ-400 spectrometer with TMS as an internal standard and $CDCl_3$, DMSO-d_6 as the solvents. The chemical shifts were expressed in parts per million (δ ppm). Coupling constants (J) were measured in Hz. MS were taken by Bruker Apex IV FTMS with methanol as the solvent. Melting points were measured on an X-4 apparatus without correction. Silica gel H was used for the column chromatography and Silica gel GF254 was used for TLC plates, which were used to monitor the reactions. CH_3CN and acetone were dried with $CaCl_2$, other chemicals were purchased as reagent grade and used without further purification.

General procedure for the syntheses of compounds **5a-e:** 6-substituted benzyl-2-thioxouracil(compound **4**, 0.47mmol) and K_2CO_3(0.94mmol) were dissolved in DMSO(2 mL) and stirred at room temperature, then the corresponding substituted alkyl bromide(0.47mmol) was added to the solution followed by stirring for 12 h. 7 mL H_2O was added and then the pH of solution was adjusted to 3 with 2N hydrochloric acid. After the addition of 10 mL ethyl acetate, the solution was stirred for another 15 min. Ethyl acetate 20 mL×3 was used to extract the aqueous layer and the organic phases were combined, dried over $MgSO_4$ and concentrated at reduced pressure with a rotary evaporator. The crude product was purified with silica gel H, and EtOAc–petroleum ether was used as the eluting solvent, to yield the target products **5a-e**.

General procedure for the syntheses of compounds **6a-e:** Compound **5a-e** (0.30mmol) was dissolved in DMF (3 mL) and stirred at 0°C, then NIS (0.36mmol) was added to this solution followed by stirring for 5h. The product was purified with silica gel H, and EtOAc-petroleum ether was used as the eluting solvent, to yield products **6a-e**.

4.2 Biology

4.2.1In vitro cytotoxicity study of target compounds.

Cytotoxicity of potent compounds was assessed by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay via measuring the amount of HepAD38 (stable expression of HBV) cells after exposure to compounds for 7 days. Briefly, logarithmically growing cells were seeded in 96-well culture plates at 1× 10^5 cells/mL (200μL/well). Twenty-four hours later, cells were treated with various concentrations of each compound (50μM, 100μM, 200μM). Cells treated with culture medium containing 0.4% DMSO were used as negetive control. Media were changed every 2 days. After 7 days, 20μL of MTT solution was added to per well and incubation was allowed to continue for another 4h. Then, the culture medium was discarded and 150μL of DMSO was added to each well to solubilize the formazan. The absorbance (A) of the formazan at 490 nm was measured by using an automatic plate reader. The survival ratio of HepAD38 cells (%) was calculated using the following formula: 1- (A490 of experiment group/A490 of negative control) × 100%.

4.2.2Detection of extracellular HBV DNA

Inhibition of the synthesized target compounds of extracellular virus was determined in the HepAD38 cell line. Briefly, HepAD38 cells were plated onto 48-well plates. After 24h, the medium was removed and was replaced with medium containing test compound. Two days after the addition of compound, the medium was removed, the cells were washed with warmed (37°C) phosphate-buffered saline (PBS),and fresh medium containing compound was added. 72h later,the medium was collected,boiled for 10min and clarified at 12000 rpm for 10min. The quantity of viral DNA in the medium was determined by Quantitative Real-time PCR(qRT-PCR). The forward primer was 5′-CAACCTCCAATCACTCACCAAC-3′ and the reverse primer was 5′-ACGGGCAACATACCTTGGTAG-3′. PCR reactions were performed on Lightcycler 480II Realtime PCR Detection System. Melt curve analysis was conducted according to the manufacturer's instructions to verify that single PCR product. The Ct values generated from the DMSO-treated cells were used as control.

4.2.3Determination of viral HBsAg and HBeAg antigens

HBV antigen of target compounds were evaluated in cultured Huh7 cell line. Two days after the treatment of test compounds, the medium was removed, the cells were washed with warmed (37°C) phosphate-buffered saline (PBS), and fresh medium containing compound was added. After 7 days, cell culture media were collected. For the HBV antigens detection, a total of 100μL cell culture media were used for each test by using time-resolved fluoroimmunoassay (TRFIA) according to manufacturer's instructions (SY60108A and SY60105A; PerkinElmer).

4.2.4General Method for intracellular HBV DNA and total RNA inhibition assays.

The Huh-7 cells were seeded in 24-well plates. Twenty-four hours later, cells were transiently transfected with 1.2 xHBV plasmid and vector control using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) following the Manufacturer's recommendations. Compounds with different doses were added in cell culture medium 6h later. After treatment with compound for 48h, cells were harvested and the intracellular HBV DNA and total RNA were measured. Briefly, DNA was extracted from Huh-7 cells by using a commercially available DNA extraction kit (QIAamp DNA Mini Kit, Qiagen, Germany). Intracellular HBV DNA analysis was performed by qRT-PCR as previously described for extracellular HBV DNA detection. Meanwhile, Total RNA was extracted from the TRIzol reagents (Invitrogen, Carlsbad, Calif) from the Huh-7cells according to the manufacturer'sinstructions. The isolated RNA was first subjected to reverse transcription and cDNA was prepared by SuperScript® III RT-PCR kits(Invitrogen, Grand Island, NY, USA). The cDNA was then quantified using the Real-time PCR assay which was carried out to measure the expression level of 3.5kb transcripts of HBV. The house keeping gene ACTB was used as an internal control. The Primer sequences for ACTB amplification were as follows: the forward primer was 5′-CTACAGCTTCACCACCACGG-3′ and the reverse primer was 5′-TCAGGCAGCTCGTAGCTCTTC-3′. The relative expression level of HBV RNA was calculated by the $2^{-\Delta CT}$.

5.Acknowledgments

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Graphical abstract

Pyrimidone derivatives have been synthesized and evaluated as anti-HBV agents, two compounds exhibited more potent than lamivudine.