Accepted Manuscript

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PII: S0223-5234(16)30609-2

DOI: 10.1016/j.ejmech.2016.07.048

Reference: EJMECH 8766

To appear in: *European Journal of Medicinal Chemistry*

Received Date: 28 May 2016

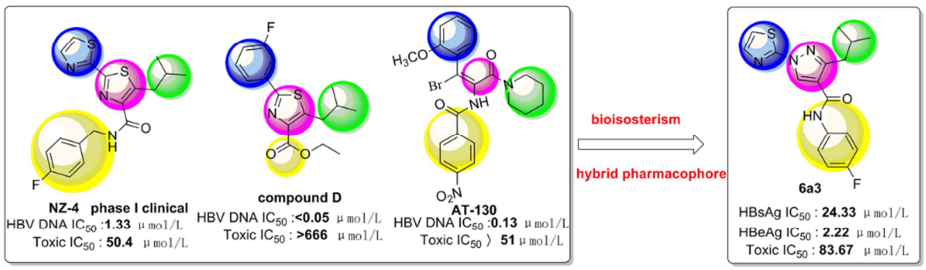
Revised Date: 18 July 2016

Accepted Date: 20 July 2016

Please cite this article as: H. Jia, F. Bai, N. Liu, X. Liang, P. Zhan, C. Ma, X. Jiang, X. Liu, Design, synthesis and evaluation of pyrazole derivatives as non-nucleoside hepatitis B virus inhibitors, *European Journal of Medicinal Chemistry* (2016), doi: 10.1016/j.ejmech.2016.07.048.

Graphical abstract

A series of novel pyrazole derivatives were identified as non-nucleoside HBV inhibitors via bioisosterism and pharmacophore hybrid strategy.



Design, Synthesis and Evaluation of Pyrazole Derivatives as Non-nucleoside Hepatitis B Virus Inhibitors

Haiyong Jia[1], Fuxiang Bai[2], Na Liu[1], Xiaohong Liang[2], Peng Zhan[1], Chunhong Ma[2], Xuemei Jiang[3] and Xinyong Liu[1,*]

[1] *Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (Ministry of Education), School of Pharmaceutical Sciences, Shandong University, 44 West Culture Road, 250012, Jinan, Shandong, PR China*

[2] *Department of Immunology, Key Laboratory for Experimental, Teratology of Ministry of Education, Shandong Provincial Key Laboratory of Infection and Immunology, Shandong University School of Medicine, Jinan 250012, Shandong Province, China.*

[3] *Department of Hepatic Diseases, Jinan Infectious Disease Hospital, Jingshi Road, 173，250021, Jinan, Shandong, P. R. China*

**Corresponding author.* Tel.: +86 531 88380270; fax: +86 531 88382731.

E-mail address: xinyongl@sdu.edu.cn.

Abstract

In continuation of our efforts toward the discovery of potent non-nucleoside hepatitis B virus (HBV) inhibitors with novel structures, we have employed bioisosterism and hybrid pharmacophore-based strategy to explore the chemically diverse space of bioactive compounds. In this article, the original thiazole platform was replaced with pyrazole scaffold to yield the optimal pharmacophore moieties in order to generate novel non-nucleoside HBV inhibitors with desirable potency. Some of the new compounds were able to inhibit HBV activity in the low micromolar range. In particular, compound **6a3** displayed the most potent activity against the secretion of HBsAg and HBeAg with IC_{50} of 24.33 μM and 2.22 μM, respectively. The preliminary structure-activity relationship (SAR) of this new series of compounds was investigated, which may help designing more potent molecules.

Keywords: HBV; Bioisosterism; Hybrid pharmacophore-based; Non-nucleoside; SAR.

1. Introduction

Viral hepatitis type B, referred to as Hepatitis B, is a serious infectious disease caused by the hepatitis B virus (HBV). Long-term development of Hepatitis B can lead to acute or chronic viral hepatitis, severe hepatitis, liver cirrhosis (LC) and hepatocellular carcinoma (HCC)[1]. According to the report of World Health Organization (WHO), about 240 million people worldwide had been involved in chronic HBV infection[2], and more than 780,000 people die every year[3]. Due to the high incidence, long course and difficulty to cure, hepatitis B has become a major disease which seriously affect people's health and social development, therefore research of effective drugs of HBV has become the top priority [4]. China is a high prevalence country of hepatitis B with about 90 million HBV carriers and an average 28 million people have chronic hepatitis B (CHB).

HBV is a member of hepadnaviridae, whose genome is partially double-stranded circular. But its replication process possesses the same characteristics as RNA retroviruses replication process, which including: adsorption and fusion to the target cells, DNA repair and transcription, the translation and reverse transcription of progenome RNA, viral particle assembly and budding, etc[5]. Based on the understanding of HBV life cycle and molecular biology, some drugs have been developed for the treatment of CHB. Those currently used drugs mainly include interferon, immunomodulatory drugs, and DNA polymerase inhibitors. Despite the dual role of immunomodulation and anti-virus of α-interferon (IFN-α) [6], it is effective only to 30%-40% of patients, moreover adverse reactions limit its clinical application. Thymosin-α1 (Tα1) [7] and other immunomodulatory drugs can improve the body's specific immunity to HBV but lack of pertinency, so they can be used only as adjuvant drugs or as part of a combined treatment with other HBV drugs. Five nucleoside/nucleotide HBV DNA polymerase inhibitors (lamivudine, adefovir dipivoxil, entecavir, telbivudine and tenofovir) (**Figure 1**) are widely used in clinic[8]. However, because of the genetic heterogeneity of HBV genome, the virus can easily develop resistance to this kind of drugs. Therefore, discovery and

development of novel anti-HBV inhibitors with improved potency, low toxicity, or novel modes of actions is undoubtedly essential to combat the HBV infection[9].

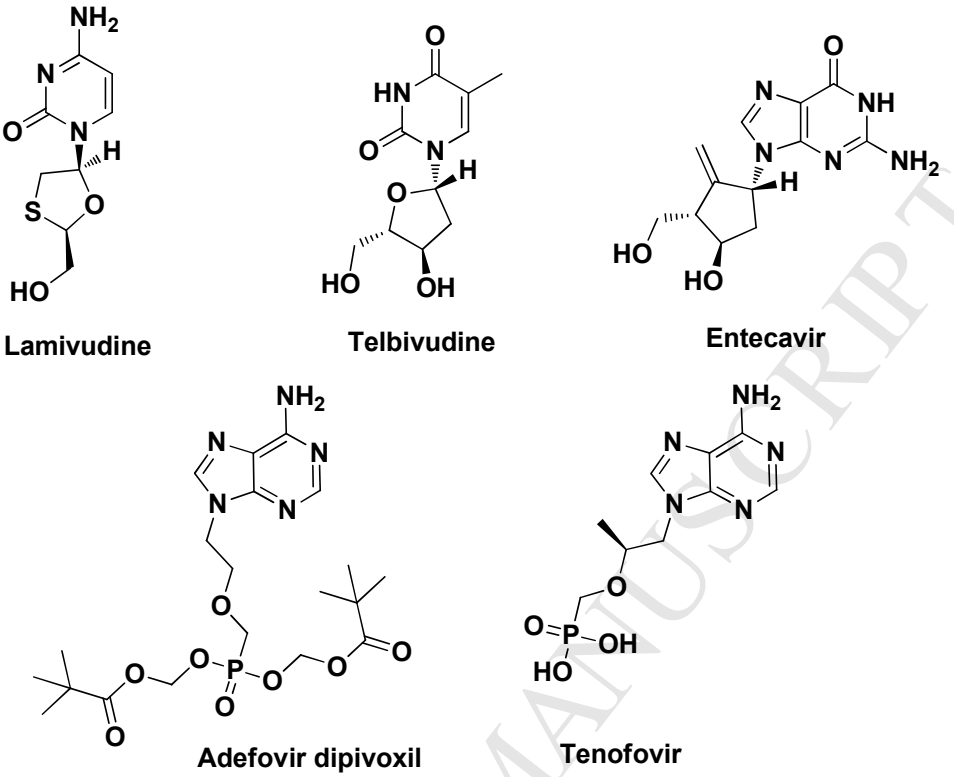


Figure 1. Five nucleoside/nucleotide drugs approved by US FDA for HBV treatment.

Previously, Chen and co-workers first reported that leucamide A (**Figure 2**) isolated from the Australian marine sponge Leucetta microraphis, a cyclic heptapeptide containing a mixed 4,2-bisheterocycle tandem pair showed poor antiviral activity[9]. They synthesized a library of 4,2-bisheterocycle tandem derivatives, of which compound B (IC_{50}:76.4 μmol/L) (**Figure 2**) showed moderate activity against HBV DNA replication. Compound B is further optimized to afford compound C (IC_{50}: 1.1 μmol/L) (**Figure 2**) with improved activity[10]. In 2014, Li *et al*. reported that a new leucamide A derivative isothiafludine (NZ-4) (**Figure 3**) bearing bis-heterocycle tandem pairs suppressed intracellular HBV replication in HepG2.2.15 cells with an IC_{50} value of 1.33 μM. NZ-4 could inhibit HBV DNA replication by interfering with the interaction between HBcAg and pgRNA in the capsid assembly process, and provides a new therapeutic strategy to combat HBV infection[11]. Moreover, it is noteworthy that compound D (**Figure 3**), a NZ-4 analogue, have more potent activity against HBV replication[12]. Besides, phenylpropenamide AT-130

(**Figure 3**) also has potent activity against HBV DNA replication with IC_{50} value of 0.13 μM, which successfully decreased HBV production by blocking RNA packaging and producing apparently abnormal capsids that lacked genetic material[13, 14].

Generally, the structure of these compounds have four fragments (shown in different color, **Figure 3**), including the core domain (pink), a five- or six-membered aromatic ring (blue), the hydrophobic part (green), and the different amides and esters (yellow). In addition, with the aim of exploring novel structural motifs and establishing structure-activity relationships, the thiazole core of compound C was replaced with imidazole as a potential bioisosteric moiety, resulting in moderate anti-HBV activity[10]. Therefore, the five-membered heterocycle portion of these inhibitors could be acting as versatile building blocks to introduce different new functional groups and to anchor these groups into the optimal space for binding[15].

To investigate the differences in the electronic and conformational contribution of the five-membered heterocyclic moiety to the anti-HBV potency, herein, in this paper, two different series of pyrazole derivatives were designed via the bioisosterism and hybrid pharmacophore-based strategy, in which the thiazole core was replaced by its isostere pyrazole to find more potent anti-HBV inhibitors.

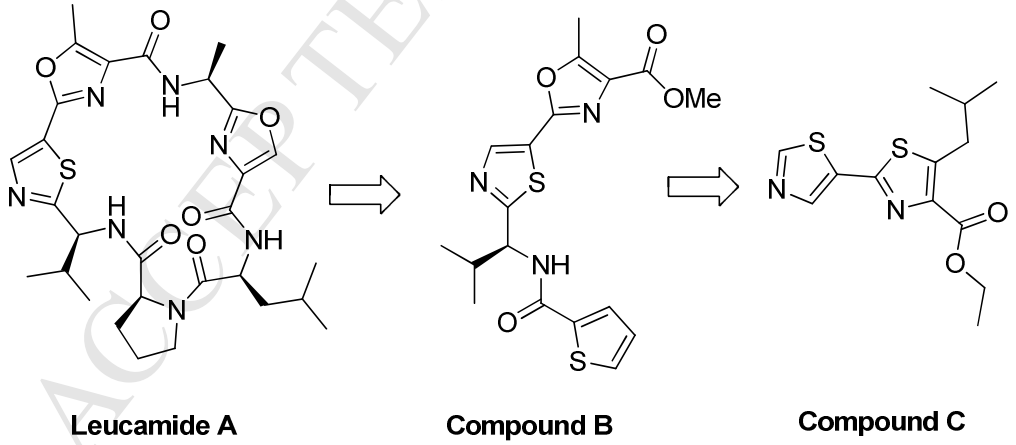


Figure 2. The structures of the anti-HBV natural product Leucamide **A** and its derivatives compound **B**, compound **C** obtained by structural simplification.

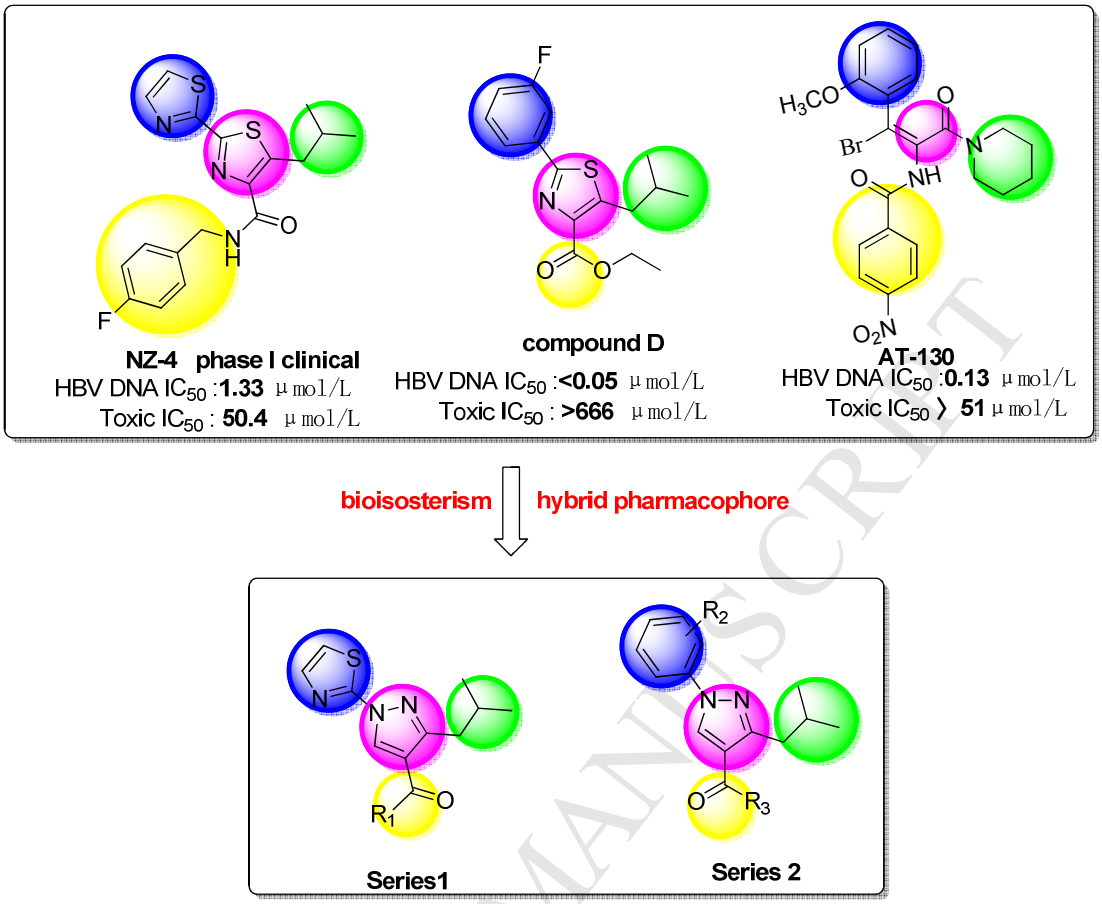


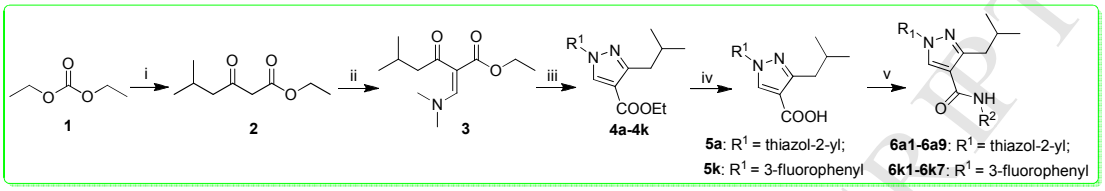
Figure 3. The structures of three lead compounds and the newly designed pyrazole series via bioisosterism and pharmacophore hybrid strategy.

2. Results and discussion

2.1. Chemistry

The synthetic route of the target compounds **6a1-6a9 and 6k1-6k7** was shown in **Scheme 1**. The key intermediate (*E*)-ethyl 2-((dimethylamino)methylene)-5-methyl-3-oxohexanoate (**3**) was prepared from the intermediate ethyl 5-methyl-3-oxohexanoate (**2**) by condensation reaction with 1,1-dimethoxy-*N*,*N*-dimethylmethanamine under 100℃.[16] The intermediate **2** was achieved from the commercially available diethyl carbonate (**1**) and 4-methylpentan-2-one by substitution reaction. Intermediate **3** was converted to **4a-4k** with different substituted hydrazines or hydrazines hydrochloride. Then, **4a** and **4b** are hydrolyzed to intermediate **5a** and **5b** correspondingly by hydrolysis reaction in

the solution of sodium hydroxide, water, ethanol and tetrahydrofuran under 50 ℃. Treatment of intermediate **5a** or **5b** with different substituted amines by the reaction gave the target compound **6a1-6a9** or **6k1-6k7**, respectively. Both analytical and spectral data of all the synthesized compounds are in full agreement with the proposed structures.



Scheme 1. Reagents and conditions: (i) 4-methylpentan-2-one, NaH, AcOH, THF; (ii) 1,1-dimethoxy-N,N-dimethylmethanamine, 80℃ (iii) EtOH, Et_3N, R^1-$NHNH_2$; (iv) NaOH, H_2O, THF, EtOH; (v) R^2-NH_2, DMF, HATU, Et_3N.

2.2. Biological activity

All the synthesized compounds were evaluated for (in vitro) their anti-HBV activity (HBeAg, HBsAg, DNA) and cytotoxicity in HepG2.2.15 cells (human HBV transgenic hepatocellular carcinoma cells) using standard ELISA, PCR and cell counting kit-8(CCK-8) method. The concentration of compound required for 50% inhibition of HBeAg, HBsAg secretion or DNA replication was defined as IC_{50} and the concentration of compound that induced the death of the HepG2.2.15 cells cultures by 50% was defined as CC_{50}. Selectivity index (SI) was determined as the CC_{50}/IC_{50} value. Lamivudine was used as positive standards.

2.2.1 Cytotoxicity

The cytotoxicity of these new pyrazole derivatives (4a-4j, 6a1-6a9 and 6k1-6k7) was measured by cell counting kit-8(CCK-8) method in HepG2.2.15 cell line. As shown in **Table 1**, most of these pyrazole analogs exhibited low toxicity as positive drug lamivudine except compound **4d** and **4f**. It was shown that electron-withdrawing group of R^1may improve the toxicity of the inhibitors.

2.2.2 Inhibitory effect on HBsAg and HBeAg secretion

To identify the effects of different polarized substituents on position R^1, ten ethyl 3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxylate derivatives (**4a-4j**) were synthesized. However, as listed in **Table 1**, most compounds showed poor antiviral activities. The 4-methoxyphenyl, 3-methoxyphenyl and 2-fluorophenyl substituent (**4e**, **4g** and **4j**) with less hydrophilic property indicated that lipophilic property was an important determinant for the anti-HBV activity.

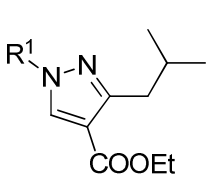
To identify the effects of the substituents in amide position, two subseries of derivatives **6a1-6a9** and **6k1-6k7** were synthesized and assessed for anti-HBV activity (**Table 1**). Compounds **6a1-6a9** with different aromatic substituents on amide position were first prepared and the pharmaceutical profiles were tested. Compared to compounds **6a8** and **6a9**, **6a6** showed more potent activity against the secretion of HBeAg with IC_{50} value 95.65 μmol/L, which is less potent than compound **6a1**. These results suggested that hydrophobic group was important to the antiviral activity. Besides, compound **6a4** bearing a trifluoromethyl moiety exhibited no antiviral activity. Additionally, the fluorobenzyl derivatives **6a1**, **6a2** and **6a7** displayed comparable activities against the secretion of HBsAg with the IC_{50} of 44.48 μmol/L, 50.12 μmol/L and 58.80 μmol/L, respectively. However, they demonstrated in opposite order (**6a1<6a2<6a7**) for the activity against the secretion of HBeAg with the IC_{50} of 31.43 μmol/L, 26.00 μmol/L and 17.25 μmol/L, respectively. In particular, compound **6a5** have more potent activity against the secretion of HBsAg and HBeAg than compound **6a1** and **6a2**, which suggested that their activity is controlled by the presence of fluoro atom at different positions of the benzyl group.

Furthermore, 4-fluorophenyl group was better than 4-fluorobenzyl group as compound **6a3** and **6k3** possessed more potent anti-HBV activity than compound **6a1** and **6k1**, respectively. Also, compound **6a1** and **6a3** were more potent inhibitors than compound **6k1** and **6k3**, which showed that thiazol-2-yl group was better than 3-fluorophenyl group for the activity against the secretion of HBsAg and HBeAg.

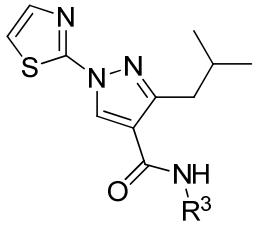
In general, the newly designed pyrazole derivatives had more potent activities against the secretion of HBsAg and HBeAg than positive drug lamivudine, but only compound **6k3** showed slightly activity against HBV DNA replication. These results

would provide valuable information for further modification through bioisosterism and pharmacophore hybrid to find more potent anti-HBV inhibitors.

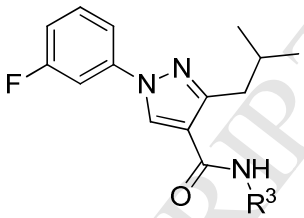
Table 1. Anti-HBV activity, cytotoxicity and selectivity indices of pyrazole derivatives (**4a-4j**, **6a1-6a9** and **6k1-6k7**) and positive drug lamivudine.



4a-4j



6a1-6a9



6k1-6k7

Compound	R^1/R^3	CC_{50}[a] (μmol/L)	HBsAg		HBeAg	
			IC_{50}[b] (μmol/L)	SI[c]	IC_{50}[b] (μmol/L)	SI[c]
4a	thiazol-2-yl	>100	>100	—	>100	—
4b	3-nitrophenyl	59.3	63.3	0.94	59.4	0.94
4c	4-COOH-Ph	>100	>100	—	>100	—
4d	3-COOH-Ph	7.1	41.9	0.17	47.9	0.15
4e	4-methoxyphenyl	77.08	68.53	1.12	71.57	1.08
4f	2-nitrophenyl	4.85	49.26	0.10	<6.25	>0.78
4g	3-methoxyphenyl	70.49	63.34	1.11	70.62	1
4h	4-nitrophenyl	>100	>100	—	>100	—
4i	4-fluorophenyl	>100	>100	—	>100	—
4j	2-fluorophenyl	>100	94.84	1.05	82.32	1.21
6a1	4-fluorobenzyl	>100	44.48	>2.25	31.43	>3.18
6a2	2-fluorobenzyl	>100	50.12	>2	26.00	>3.85
6a3	4-fluorophenyl	83.67	24.33	3.44	2.22	37.69

6a4	4-(trifluoromethyl)benzyl	>100	>100	—	>100	—
6a5	2,4-difluorobenzyl	83.60	41.82	2	8.84	9.46
6a6	4-methoxybenzyl	>100	>100	—	95.65	>1.05
6a7	3-fluorobenzyl	>100	58.80	1.7	17.25	5.80
6a8	4-aminobenzyl	>100	>100	—	>100	—
6a9	4-hydroxybenzyl	>100	>100	—	86.15	>1.16
6k1	4-fluorobenzyl	>100	>100	—	>100	—
6k2	benzyl	85.89	38.55	2.23	38.49	2.23
6k3	4-fluorophenyl	36.21	25.94	1.4	24.69	1.47
6k4	(4-methoxybenzyl)oxy	67.94	57.64	1.18	44.81	1.52
6k5	NH-(4-fluorophenyl)	45.44	51.90	—	34.01	1.34
6k6	pyridin-4-ylmethyl	>100	>100	—	71.17	1.41
6k7	tert-butyl 4-methyl--piperidine-1-carboxylate	>100	>100	—	>100	—
Lamivudine		>100	>100	—	>100	—
NZ-4[d]		51.47	34.69	1.48	31.56	1.63

[a]CC_{50}: Concentration required to reduce the viability of mock-infected cells by 50%.

[b]IC_{50}: Concentration of compound required for 50% inhibition of HBeAg or HBsAg secretion.

[c]SI: Selectivity index, the ratio of CC_{50}/IC_{50}.

[d]NZ-4： The values for NZ-4 are from single determinations.

2.2.3 Inhibitory effect on HBV DNA replication

The inhibitory effect on HBV DNA replication of some target compounds was screened in the HepG2.2.15 cell line using lamivudine (LAM) as a positive control. After the treatment of the cells with the test compounds at the concentration of 100

μM for 8 days, and then extra cellar HBV DNA levels were quantified by real-time FQ-PCR. As shown in the **fig. 4**, most of pyrazole derivatives have relative inhibitory activity against HBV DNA replication.

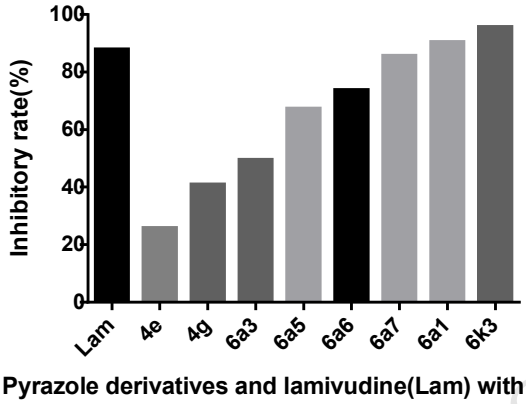


Fig. 4. Inhibitory effect of pyrazole derivatives on HBV DNA level. HepG2.2.15 cells were cultured in the presence of pyrazole derivatives and lamivudine at concentrations of 100 μM for 8 days, and then extra cellar HBV DNA levels were quantified by real-time FQ-PCR.

2.3. In silico prediction of physicochemical properties[17]

Furthermore, some physicochemical properties of **6a1**, **6a3**, **6a5** and **6k3** were predicted for their compliance with the Lipinski's rule of five using free online software (http://www.molinspiration.com/). As shown in **Table 2**, **6a1**, **6a3**, **6a5** and **6k3** compounds conformed well to the Lipinski's rule of five. Overall, compound **6a3** exhibited potent anti-HBV activity with low toxicity and reasonable physicochemical properties, and thus could be considered as a promising candidate for further development.

Table 2. Prediction of physicochemical properties[a] of **6a1**, **6a3**, **6a5** and **6k3**

Compound	nViol	MW	natoms	miLogP	nON	nOHNH	Nrotb	TPSA	MV
Range		<500		<5	<10	<5	<=10	<140	
6a1	0	385.4	25	3.36	5	1	6	59.82	314.5
6a3	0	344.4	24	3.66	5	1	5	59.82	297.6
6a5	0	376.4	26	3.45	5	1	6	59.82	319.4
6k3	0	355.4	26	4.54	4	1	5	46.92	316.1
NZ-4	0	377.5	25	3.18	4	1	6	54.35	326.1

AT-130	0	488.3	31	4.04	8	1	6	104.47	386.0

[a] nViol: no. of violations; natoms: no. of atoms; miLogP: molinspiration predicted LogP; MW: molecular weight; nON: no. of hydrogen bond acceptors; nOHNH: no. of hydrogen bond donors; nrotb: no. of rotatable bonds; TPSA: topological polar surface area; MV: molar volume.

3. Conclusions

In summary, the present work is an extension of our ongoing efforts toward the development and the identification of new molecules with anti-HBV activity. In the present investigation, the bioisosterism and hybrid pharmacophore-based drug design led to the identification of pyrazole derivatives with potency against HBV activity. Among them, compound **6a3** was identified as the most promising candidate with favorable inhibitory activity against the secretion of HBsAg and HBeAg with IC_{50} of 24.33 μmol/L and 2.22 μmol/L, respectively. Preliminary SAR results for the newly synthesized congeners are presented, providing insights for discovery of more potent anti-HBV inhibitors with diverse structures.

4. Experimental

4.1. Chemistry

All melting points were determined on a micro melting point apparatus and are uncorrected. ^{1}H NMR spectra were obtained on a Brucker Avance-400 NMR spectrometer in the indicated solvents. Chemical shifts are expressed in δ units and TMS as internal reference. Mass spectra were taken on a LC Autosampler Device: Standard G1313A instrument. TLC was performed on Silica Gel GF254 for TLC (Merck) and spots were visualized by irradiation with UV light (254 nm). Flash column chromatography was performed on column packed with Silica Gel 60 (200–300 mesh). Solvents were reagent grade and, when necessary, were purified and dried by standard methods. Concentration of the reaction solutions involved the use of rotary evaporator at reduced pressure.

4.1.1. General procedure for the synthesis of (E)-ethyl 2-((dimethylamino)methylene)-5-methyl-3-oxohexanoate (3)

The commercially available 4-methylpentan-2-one was slowly added to the solution of 60% NaH (480 mg, 11.98 mmol) in anhydrous tetrahydrofuran (15 mL) at 0 ℃ with stirring. After half an hour diethyl carbonate (**1**) (1.77 g, 14.98 mmol) was added to the mixture solution and was stirred at 60 ℃ for 4 h. The reaction mixture was added to the 50 mL ice cold water and neutralized with 1.5 mL CH_3COOH at about 5 ℃. The mixture was extracted with ethyl acetate for three time and the combined was washed by 10% Na_2CO_3 and H_2O. The product ethyl 5-methyl-3-oxohexanoate (**2**) was obtained as brown oil by removing ethyl acetate layer under reduced pressure[18], which is directly added to 10 mL 1,1-dimethoxy-*N*,*N*-dimethylmethanamine and stirred for overnight under 100 ℃. Upon completion of the reaction, the solvent was evaporated, giving the desired compound **3** as an orange oil in good yield without further purification[19].

4.1.2. General procedure for preparation of compounds 4a–4k

To the mixture solution of (E)-ethyl 2-((dimethylamino)methylene)-5-methyl-3-oxohexanoate (**3**) (335 mg，1.47 mmol) and Et_3N (622 mg，6.14 mmol) in ethanol (6 mL) were added with hydrazine or hydrazine hydrochloride (1.23 mmol). The reaction mixture was stirred at room temperature overnight. Upon completion of the reaction, the solvent was evaporated, leaving a residue which was treated with ethyl acetate (30 mL) and washed with water (3 x 30 mL). The organic layer was dried over anhydrous sodium sulfate, and then the solvent was removed under vacuum. The residue was chromatographed on silica gel using ethyl acetate and petroleum ether. Pure fractions were collected and concentrated, giving the desired compounds (**4a–4k**) in good yield.

4.1.2.1. Ethyl 3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxylate (4a)

White powder, yield: 25%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm:8.02 (s, 1H), 7.60 (d, 1H, J=3.2 Hz), 7.18 (d, 1H, J=3.6 Hz), 4.33(q, 2H, J=7.2 Hz), 3.51 (d, 2H, J=7.2 Hz), 2.16~2.06 (m,1H), 1.38 (t, 3H, J=7.2 Hz), 0.94 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.14, 161.89, 148.96, 143.46, 140.40, 117.18, 115.17, 60.25, 33.02, 28.89, 22.13, 14.35; EI-MS: 280.3 $[M+H]^+$.

4.1.2.2. Ethyl 3-isobutyl-1-(3-nitrophenyl)-1H-pyrazole-4-carboxylate (4b)

Yellow oil, yield: 45%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 8.35~8.33 (m, 2H), 8.09 (s, 1H), 7.80 (d, 1H, J=8.0 Hz), 7.72 (t, 1H, J=8.0 Hz), 4.34 (q, 2H, J=7.2 Hz), 2.98 (d, 2H, J=7.2 Hz), 1.90~1.79 (m, 1H), 1.39 (t, 3H, J=7.2 Hz), 0.77 (d, 6H, J=6.8 Hz). 13C NMR (100 MHz, CDCl3): 163.32, 148.58, 147.76, 142.95, 140.41, 132.02, 130.33, 123.52, 121.32, 113.81, 77.35, 77.24, 77.04, 76.72, 60.21, 33.36, 29.09, 22.22, 14.39; EI-MS: 318.5 $[M+H]^+$.

4.1.2.3. 4-(4-(ethoxycarbonyl)-3-isobutyl-1H-pyrazol-1-yl)benzoic acid (4c)

White powder, yield: 50%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 9.84 (s, 1H), 8.27 (d, 2H, J=8.8 Hz), 8.11 (s, 1H), 7.56 (d, 2H, J=8.8 Hz), 4.34 (q, 2H, J=7.2 Hz), 2.99 (d, 2H, J=7.2 Hz), 1.86~1.79 (m, 1H), 1.39 (t, 3H, J=7.2 Hz), 0.75 (d, 6H, J=6.8 Hz); EI-MS: 317.5 $[M+H]^+$.

4.1.2.4. 3-(4-(ethoxycarbonyl)-3-isobutyl-1H-pyrazol-1-yl)benzoic acid (4d)

White powder, yield: 52%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 9.80 (s, 1H), 8.21 (dd, 1H, J_1=1.6 Hz, J_2=7.2 Hz), 8.15 (s, 1H), 8.10 (s, 1H), 4.34 (q, 2H, J=7.2 Hz), 2.94 (d, 2H, J=7.6 Hz), 1.88~1.81 (m, 1H), 1.39 (t, 3H, J=7.2 Hz), 0.76. ^{13}C NMR (100 MHz, CDCl3): 170.08, 163.55, 147.77, 142.47, 139.57, 131.49, 130.79, 130.52, 129.68, 127.88, 113.25, 77.35, 77.24, 77.03, 76.71, 60.12, 33.36, 28.94, 22.23, 14.40; EI-MS: 317.5 $[M+H]^+$.

4.1.2.5. Ethyl 3-isobutyl-1-(4-methoxyphenyl)-1H-pyrazole-4-carboxylate (4e)

Yellow oil, yield: 32%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 8.03 (s, 1H), 7.29 (dd, 2H, J_1=2.4 Hz, J_2=6.8 Hz), 6.99 (dd, 2H, J_1=2.4 Hz, J_2=6.8 Hz), 4.32 (q, 2H, J=7.2 Hz), 3.87 (s, 1H), 2.86 (d, 2H, J=7.2 Hz), 1.90~1.80 (m, 1H), 1.37 (t, 3H, J=7.2 Hz), 0.75 (d, 6H, J=6.8 Hz), EI-MS: 303.5 $[M+H]^+$.

4.1.2.6. Ethyl 3-isobutyl-1-(2-nitrophenyl)-1H-pyrazole-4-carboxylate (4f)

Yellow oil, yield: 52%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 8.10 (dd, 1H, J_1=0.8 Hz, J_2=8.0 Hz), 8.05 (s, 1H), 7.78 (dt, 1H, J_1=1.2 Hz, J_2=7.6 Hz), 7.68 (dt, 1H, J_1=1.2 Hz, J_2=7.6 Hz), 7.54 (dd, 1H, J_1=0.8 Hz, J_2=7.6 Hz), 4.33 (q, 2H, J=7.2 Hz), 2.83 (d, 2H, J=7.6 Hz), 1.89~1.79 (m, 1H), 1.38 (t, 3H, J=7.2 Hz), 0.81 (d, 6H, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 163.34, 148.59, 146.03, 143.16, 133.50, 132.53, 130.42, 129.77, 125.64, 113.52, 77.36, 77.05, 76.73, 60.08, 33.45, 28.43, 22.23, 14.36;

EI-MS: 318.5 $[M+H]^+$.

4.1.2.7. Ethyl 3-isobutyl-1-(3-methoxyphenyl)-1H-pyrazole-4-carboxylate (4g)

Yellow oil, yield: 47%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.39 (t, 1H, J=8.4 Hz), 7.00 (dd, 1H, J_1=1.6 Hz, J_2=8.4 Hz), 6.98 (dd, 1H, J_1=1.6 Hz, J_2=8.4 Hz), 6.92 (t, 1H, J=2.0 Hz), 4.32 (q, 2H, J=7.2 Hz), 3.85 (s, 3H), 2.92 (d, 2H, J=7.2 Hz), 1.89~1.82 (m, 1H), 1.38 (t, 3H, J=7.2 Hz), 0.76 (d, 6H, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 163.69, 160.20, 147.55, 141.98, 140.27, 129.92, 118.58, 114.93, 112.81, 112.12, 77.35, 77.24, 77.03, 76.72, 59.95, 55.58, 33.38, 28.74, 22.24, 14.41; EI-MS: 303.5 $[M+H]^+$.

4.1.2.8. Ethyl 3-isobutyl-1-(4-nitrophenyl)-1H-pyrazole-4-carboxylate (4h)

Yellow oil, yield: 37%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 8.39 (dd, 2H, J_1=2.0 Hz, J_2=6.8 Hz), 8.09 (s, 1H), 7.64 (dd, 2H, J_1=2.0 Hz, J_2=6.8 Hz), 7.27 (s, 1H), 4.34 (q, 2H, J=7.2 Hz), 3.01 (d, 2H, J=7.2 Hz), 1.85~1.78 (m, 1H), 1.39 (t, 3H, J=7.2 H), 0.75 (d, 6H, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 163.28, 147.78, 147.34, 144.53, 143.15, 126.68, 124.82, 114.13, 77.35, 77.24, 77.04, 76.72, 60.23, 33.36, 29.09, 22.18, 14.38; EI-MS: 318.5 $[M+H]^+$.

4.1.2.9. Ethyl 1-(4-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxylate (4i)

Yellow oil, yield: 56%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm:8.03 (s, 1H), 7.38~7.35 (m, 2H), 7.21~7.17 (m, 2H), 4.32 (q, 2H, J=7.2 Hz), 2.88 (d, 2H, J=7.2 Hz), 1.87~1.80 (m, 1H), 1.38 (t, 3H, J=7.2 Hz), 0.75 (d, 6H, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 163.75, 163.60, 161.27, 147.65, 142.11, 135.42, 135.39, 128.41, 128.32, 116.37, 116.14, 112.86, 77.34, 77.23, 77.02, 76.71, 59.97, 33.34, 28.76, 22.20, 14.39; EI-MS: 291.4 $[M+H]^+$.

4.1.2.10. Ethyl 1-(2-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxylate (4j)

Yellow oil, yield: 58%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 8.09 (s, 1H), 7.52~7.46 (m, 1H), 7.44~7.39 (m, 1H), 7.31~7.24 (m, 2H), 4.33 (q, 2H, J=7.2 Hz), 2.79 (d, 2H, J=7.6 Hz), 1.90~1.80 (m, 1H), 1.38 (t, 3H, J=7.2 Hz), 0.75 (d, 6H, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 163.53, 158.33, 155.82, 149.02, 142.73, 131.29, 131.21, 129.52, 127.18, 127.06, 124.77, 124.73, 116.91, 116.72, 112.65, 77.36, 77.24, 77.04, 76.72, 59.99, 33.53, 33.51, 28.68, 22.20, 14.40; EI-MS: 291.4 $[M+H]^+$.

4.1.2.11. Ethyl 1-(3-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxylate (4k)

Yellow oil, yield: 38%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm:8.05 (s, 1H), 7.50~7.45 (m, 1H), 7.21~7.14 (m, 3H), 4.34 (q, 2H, J=7.2 Hz), 2.94 (d, 2H, J=7.6 Hz), 1.89~1.78 (m, 1H), 1.38 (t, 3H, J=7.2 Hz), 0.76 (d, 6H, J=6.8 Hz). ^{13}C NMR (100 MHz, $CDCl_3$): 163.90, 163.55, 161.43, 147.59, 142.37, 140.70, 140.60, 130.56, 130.47, 122.08, 122.05, 116.13, 115.92, 114.21, 113.97, 113.18, 77.35, 77.24, 77.03, 76.71, 60.04, 33.31, 28.85, 22.21, 14.40, EI-MS: 291.4 $[M+H]^+$.

4.1.3. General procedure for preparation of compounds 5a and 5k

Ethyl 3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxylate **(4a)** or ethyl 1-(3-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxylate **(4k)** (0.69 mmol) was added to the mixture solution of ethanol (3 mL), water (3 mL) and THF (3 mL) with NaOH (1.29 g, 32.2 mmol). The reaction mixture was stirred at 50 ℃ overnight. Upon completion of the reaction, the ethanol and THF was evaporated, leaving a residue which was neutralized with hydrochloric acid (1 mol/L) to PH=3, treated with ethyl acetate (30 mL) and washed with water (3 x 30 mL). The organic layer was dried over anhydrous sodium sulfate, and then the solvent was removed under vacuum to give the desired compounds **(5a** or **5k)** in good yield.

4.1.3.1. 3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxylic acid (5a)

White solid, yield: 87%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: :12.79 (s, 1H), 8.11 (s, 1H), 7.76 (d, 1H, J=3.6 Hz), 7.67 (d, 1H, J=3.2 Hz), 3.45 (d, 2H, J=7.2 Hz), 2.07~2.00 (m,1H), 0.88 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 164.30, 161.71, 148.11, 144.06, 140.95, 119.37, 115.92, 32.70, 28.69, 22.34; EI-MS: 252.4 $[M+H]^+$.

4.1.3.2. 3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxylic acid (5k)

White solid, yield:66%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm:8.02 (s, 1H), 7.60 (d, 1H, J=3.2 Hz), 7.18 (d, 1H, J=3.6 Hz), 4.33(q, 2H, J=7.2 Hz), 3.51 (d, 2H, J=7.2 Hz), 2.16~2.06 (m,1H), 1.38 (t, 3H, J=7.2 Hz), 0.94 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.14, 161.89, 148.96, 143.46, 140.40, 117.18, 115.17, 60.25, 33.02, 28.89, 22.13, 14.35; EI-MS: 280.3 $[M+H]^+$.

4.1.4. General procedure for preparation of compounds 6a1-6a9 and 6k1-6k8

To the mixture solution of 3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxylic acid **(5a)** or 3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxylic acid **(5k)** (0.38 mmol) in DMF (5 ml) 2-(7-Aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) (174 mg, 0.46 mmol) was added under low temperature with stirring for 10 minutes. Et_3N (77 mg, 0.76 mmol) and substituted amines (0.38 mmol) was added slowly with stirring under room temperature for 1h. Upon completion of the reaction, the mixture solution was diluted with water (125 mL), treated with ethyl acetate (3 x 30 mL) and washed with saturated NaCl (3 x 30 mL). The organic layer was dried over anhydrous sodium sulfate, and then the solvent was removed under vacuum. The residue was chromatographed on silica gel using ethyl acetate and petroleum ether. Pure fractions were collected and concentrated, giving the desired compounds **(6a1-9 and 6k1-8)** in good yield.

4.1.4.1.

N-(4-fluorobenzyl)-3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide (6a1)

White solid, yield: 72%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.80 (s, 1H), 7.60 (d, 1H, J=3.6 Hz), 7.32~7.29(m, 2H), 7.17 (d, 1H, J=3.6 Hz), 7.02 (t, 2H, J=8.4 Hz), 6.29 (s, 1H), 4.55 (d, 2H, J=5.6 Hz), 3.50 (d, 2H, J=7.2 Hz), 2.12~2.05 (m, 1H), 0.92 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.45, 162.82, 161.74, 161.01, 147.53, 140.43, 139.87, 134.12, 134.09, 133.82, 129.55, 129.47, 117.72, 117.17, 115.71, 115.49, 114.43, 77.37, 77.05, 76.74, 42.77, 32.92, 28.86, 22.12; EI-MS: 359.4 $[M+H]^+$.

4.1.4.2.

N-(2-fluorobenzyl)-3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide (6a2)

White solid, yield: 49%, MP: 101-102 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.80 (s, 1H), 7.59 (d, 1H, J=3.2 Hz), 7.40 (t, 1H, J=7.2 Hz), 7.38~7.25(m, 1H), 7.16 (d, 1H, J=3.6 Hz), 7.14~7.04 (m, 2H), 6.24 (s, 1H), 4.65 (d, 2H, J=5.6 Hz), 3.48 (d, 2H, J=7.2 Hz), 2.12~2.02 (m, 1H), 0.90 (d, 6H, J=7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 162.81, 162.35, 161.79, 159.90, 147.34, 140.40, 139.99, 130.39, 130.35, 129.45, 129.37, 125.28, 125.14, 124.40, 124.37, 117.87, 117.14, 115.55, 115.33, 37.59, 37.55, 32.93, 28.84, 22.06; EI-MS: 359.5 $[M+H]^+$.

4.1.4.3.

N-(4-fluorophenyl)-3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide (6a3)

White solid, yield: 28%, MP: 126-127 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 8.37 (t, 1H, J=8.0 Hz), 7.95 (s, 1H), 7.71 (s, 1H), 7.62 (d, 1H, J=3.6 Hz), 7.20 (d, 1H, J=3.6 Hz), 7.17~7.07 (m, 3H), 3.56 (d, 2H, J=7.2 Hz), 2.18~2.11 (m, 1H), 0.96 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 161.70, 160.77, 153.83, 151.42, 147.92, 140.45, 140.06, 126.37, 126.27, 124.70, 124.66, 124.52, 124.44, 122.05, 118.05, 117.39, 114.97, 114.78(d), 33.02, 28.92, 22.11; EI-MS: 345.4 $[M+H]^+$.

4.1.4.4.

3-isobutyl-1-(thiazol-2-yl)-N-(4-(trifluoromethyl)benzyl)-1H-pyrazole-4-carboxamide (6a4)

White solid, yield: 83%, MP: 163-164 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.81 (s, 1H), 7.61 (s, 1H), 7.59 (d, 2H, J=7.2 Hz), 7.44 (d, 2H, J=8.0 Hz), 7.17 (d, 1H, J=3.6 Hz), 6.35 (s, 1H), 4.64 (d, 2H, J=6.0 Hz), 3.51 (d, 2H, J=7.2 Hz), 2.12~2.05 (m, 1H), 0.92 (d, 6H, J=7.2 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 162.97, 161.69, 147.71, 142.43, 140.44, 139.76, 127.88 (d), 125.70, 125.66, 117.50, 117.22, 77.34, 77.03, 76.71, 42.95, 32.93, 28.86, 22.09; EI-MS: 409.5 $[M+H]^+$.

4.1.4.5.

N-(2,4-difluorobenzyl)-3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide (6a5)

White solid, yield: 92%, MP: 107-108 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.80 (s, 1H), 7.59 (d, 1H, J=3.2 Hz), 7.42~7.36(m, 1H), 7.16 (d, 1H, J=3.2 Hz), 6.87~6.79 (m, 2H), 6.27 (s, 1H), 4.59 (d, 2H, J=5.6 Hz), 3.48 (d, 2H, J=7.2 Hz), 2.10~2.03 (m, 1H), 0.90 (d, 6H, J=6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.77, 163.65, 162.87, 162.35, 162.23, 161.75, 161.30, 161.18, 159.87, 159.75, 147.39, 140.40, 139.91, 131.34, 131.28, 131.24, 131.19, 121.40, 121.36, 121.25, 121.21, 117.70, 117.16, 111.56, 111.52, 111.34, 111.31, 104.16, 103.91, 103.66, 77.34, 77.03, 76.71, 37.01, 36.98, 32.91, 28.83, 22.05; EI-MS: 377.5 $[M+H]^+$.

4.1.4.6.

3-isobutyl-N-(4-methoxybenzyl)-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide

(6a6)

White solid, yield: 96%. MP: 111-113 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.77 (s, 1H), 7.59 (d, 1H, J=3.2 Hz), 7.26 (d, 2H, J=8.4 Hz), 7.16 (d, 1H, J=2.8 Hz), 6.87 (d, 2H, J=8.4 Hz), 6.12 (s, 1H), 4.52 (d, 2H, J=5.2 Hz), 3.80 (s, 3H), 3.51 (d, 2H, J=7.6 Hz), 2.13~2.04 (m, 1H), 0.93 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 162.69, 161.81, 159.16, 147.41, 140.40, 139.91, 130.30, 129.26 (d), 117.94, 117.10, 114.19, 77.36, 77.04, 76.72, 55.32, 43.05, 32.93, 28.86, 22.12; EI-MS: 371.5 $[M+H]^+$.

4.1.4.7.

N-(3-fluorobenzyl)-3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide (6a7)

White solid, yield: 79%, MP: 117-119 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.81 (s, 1H), 7.59 (d, 1H, J=3.6 Hz), 7.29 (q, 1H, J=7.6 Hz), 7.17 (d, 1H, J=3.2 Hz), 7.10 (d, 1H, J=7.6 Hz), 7.03 (d, 1H, J=9.6 Hz), 6.97 (t, 1H, J=7.6 Hz), 6.28 (s, 1H), 4.58 (d, 2H, J=5.6 Hz), 3.51 (d, 2H, J=7.2 Hz), 2.12~2.06 (m, 1H), 0.92 (d, 6H, J=6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 164.27, 162.88, 161.82, 161.73, 147.62, 140.93, 140.86, 140.43, 139.83, 130.32, 130.23, 123.23, 123.20, 117.64, 117.18, 114.71, 114.57, 114.49, 114.36, 77.35, 77.03, 76.72, 42.94, 32.93, 28.87, 22.11; EI-MS: 359.6 $[M+H]^+$.

4.1.4.8.

N-(4-aminobenzyl)-3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide (6a8)

White solid, yield: 79%, MP: 126-128 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.75 (s, 1H), 7.59 (d, 1H, J=3.2 Hz), 7.15 (d, 2H, J=3.2 Hz), 7.13 (d, 1H, J=8.0 Hz), 6.65 (d, 2H, J=8.0 Hz), 6.06 (s, 1H), 4.46 (d, 2H, J=5.6 Hz), 3.50 (d, 2H, J=7.2 Hz), 3.42 (s, 2H), 2.14~2.06 (m, 1H), 0.92 (d, 6H, J=6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 162.64, 161.84, 147.32, 145.97, 140.38, 139.95, 129.28, 127.97, 118.04, 117.08, 115.29, 77.37, 77.05, 76.73, 43.26, 32.92, 28.86, 22.13; EI-MS: 356.5 $[M+H]^+$.

4.1.4.9.

N-(4-hydroxybenzyl)-3-isobutyl-1-(thiazol-2-yl)-1H-pyrazole-4-carboxamide (6a9)

White solid, yield: 55%, MP: 122-124 ℃. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 9.31 (s, 1H), 8.70 (s, 1H), 8.24 (s, 1H), 7.73 (s, 1H), 7.63 (s, 1H), 7.12 (d, 2H, J=7.6

Hz), 6.72 (d, 2H, J=7.6 Hz), 4.33 (d, 2H, J=4.0 Hz), 3.48 (d, 2H, J=7.2 Hz), 2.01~1.98 (m, 1H), 0.84 (d, 6H, J=6.0 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 162.28, 161.91, 156.71, 146.58, 141.59, 140.91, 130.26, 129.05, 119.07, 118.51, 115.48, 42.01, 40.62, 40.41, 40.20, 40.00, 39.79, 39.58, 39.37, 32.53, 28.71, 22.37; EI-MS: 357.4 $[M+H]^+$.

4.1.4.10. N-(4-fluorobenzyl)-1-(3-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxamide (6k1)

White solid, yield: 55%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.79 (s, 1H), 7.49~7.44 (m, 1H), 7.31 (dd, 2H, J_1=5.6 Hz, J_2=8.8 Hz), 7.19 (dd, 2H, J_1=2.0 Hz, J_2=8.0 Hz), 7.15~7.12 (m, 1H), 6.24 (s, 1H), 4.56 (d, 2H, J=6.0 Hz), 2.98 (d, 2H, J=7.6 Hz), 1.87~1.76 (m,1H), 0.75 (d, 6H, J=6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.91, 163.47, 163.33, 161.44, 161.02, 146.43, 140.77, 140.67, 138.42, 134.31, 134.28, 130.56, 130.47, 129.50, 129.42, 121.95, 121.91, 116.09, 115.88, 115.73, 115.71, 115.49, 114.08, 113.84, 77.36, 77.25, 77.04, 76.73, 42.69, 33.15, 28.78, 22.19; EI-MS: 370.4 $[M+H]^+$.

4.1.4.11. N-benzyl-1-(3-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxamide (6k2)

White solid, yield: 60%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: :7.79 (s, 1H), 7.79~7.44 (m, 1H), 7.36~7.27 (m, 5H), 7.20~7.12 (m, 3H), 6.21 (s, 1H), 4.61 (d, 2H, J=4.4 Hz), 2.99 (d, 2H, J=7.2 Hz), 1.88~1.78 (m,1H), 0.75 (d, 6H, J=6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.90, 163.27, 161.42, 146.40, 140.74, 140.64, 138.40, 130.55, 130.46, 128.79, 127.85, 127.58, 121.96, 121.93, 116.07, 115.86, 114.09, 113.85, 77.35, 77.24, 77.04, 76.72, 43.45, 33.15, 28.77, 22.20; EI-MS: 352.5 $[M+H]^+$.

4.1.4.12. 1-(3-fluorophenyl)-N-(4-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxamide (6k3)

White solid, yield: 48%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm:8.00 (s, 1H), 7.78 (s, 1H), 7.56~7.53 (m, 2H), 7.51~7.45 (m, 1H), 7.21 (dd, 2H, J_1=2.0 Hz, J_2=8.0 Hz), 7.16 (dd, 1H, J_1=2.0 Hz, J_2=9.2 Hz), 7.04 (t, 2H, J=8.4 Hz), 2.99 (d, 2H, J=7.2 Hz), 1.89~1.82 (m,1H), 0.75 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.91,

161.52, 161.43, 160.73, 158.30, 147.24, 140.40, 140.30, 138.37, 133.79, 133.76, 130.66, 130.57, 122.42, 122.34, 122.04, 122.00, 116.36, 116.15, 115.96, 115.81, 115.59, 114.17, 113.93, 77.36, 77.24, 77.04, 76.72, 33.19, 28.74, 22.21.

4.1.4.13.

1-(3-fluorophenyl)-3-isobutyl-N-((4-methoxybenzyl)oxy)-1H-pyrazole-4-carboxamide (6k4)

White solid, yield: 48%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 8.67 (s, 1H), 7.74 (s, 1H), 7.48~7.43(m, 1H), 7.35 (d, 2H, J=8.8 Hz), 7.19~7.10(m, 1H), 6.90 (d, 2H, J=8.4 Hz), 4.92 (s, 2H), 3.81 (s, 3H), 2.93 (d, 2H, J=7.6 Hz), 1.82~1.75 (m, 1H), 0.74 (d, 6H, J=6.4 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.88, 161.40, 160.07, 146.97, 140.59, 140.49, 138.74, 131.04, 130.56, 130.47, 127.40, 121.92, 121.89, 116.12, 115.91, 114.04, 114.01, 113.80, 112.79, 78.19, 77.38, 77.06, 76.74, 55.29, 33.17, 28.71, 22.17; EI-MS: 398.4 $[M+H]^+$.

4.1.4.14.

1-(3-fluorophenyl)-N'-(4-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carbohydrazide (6k5)

White solid, yield: 45%. ^{1}H NMR (400 MHz, DMSO) δppm: 10.08 (s, 1H), 8.27 (s, 1H), 7.87 (s, 1H), 7.61 (q, 1H, J=6.8 Hz), 7.48 (d, 1H, J=9.6 Hz), 7.38 (t, 2H, J=8.0 Hz), 7.00 (t, 2H, J=8.8 Hz), 6.80~6.77(m, 2H), 2.96 (d, 2H, J=7.6 Hz), 1.65~1.58 (m, 1H), 0.64 (d, 6H, J=6.4 Hz); ^{13}C NMR (100 MHz, DMSO): 163.66, 163.27, 161.21, 157.48, 155.16, 146.79, 146.27, 141.13, 141.03, 139.60, 131.59, 131.50, 122.73, 116.28, 116.08, 115.71, 115.49, 114.40, 114.07, 113.88, 113.80, 40.65, 40.44, 40.24, 40.03, 39.82, 39.61, 39.40, 32.86, 28.40, 22.43; EI-MS: 371.4 $[M+H]^+$.

4.1.4.15.

1-(3-fluorophenyl)-3-isobutyl-N-(pyridin-4-ylmethyl)-1H-pyrazole-4-carboxamide (6k6)

White solid, yield: 61%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm:8.53 (s, 1H), 7.89 (s, 1H), 7.47 (q, 1H, J=6.4 Hz), 7.28~7.14(m, 5H), 6.81 (s, 1H), 4.60 (d, 2H, J=6.0 Hz), 2.99 (t, 2H, J=7.2 Hz), 1.84~1.78 (m, 1H), 0.75 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.89, 163.63, 161.42, 149.90, 147.96, 146.73, 140.67, 140.58,

138.52, 130.60, 130.51, 122.29, 121.93, 121.90, 116.16, 115.95, 115.37, 114.07, 113.83, 77.37, 77.05, 76.73, 42.13, 33.12, 28.76, 22.18; EI-MS: 353.4 $[M+H]^+$.

4.1.4.16. Tert-butyl 4-((1-(3-fluorophenyl)-3-isobutyl-1H-pyrazole-4-carboxamido)methyl)piperidine-1-carboxylate (6k7)

White solid, yield: 60%. ^{1}H NMR (400 MHz, $CDCl_3$) δppm: 7.81 (s, 1H), 7.50~7.44(m, 1H), 7.20~7.13(m, 3H), 6.16 (t, 1H, J=6.0 Hz), 4.13 (d, 2H, J=12.8 Hz), 3.30 (d, 2H, J=5.2 Hz), 2.97 (d, 2H, J=7.6 Hz), 2.70 (t, 2H, J=12.4 Hz), 1.84~1.72(m, 4H), 1.46 (s, 9H), 1.23~1.13 (m, 2H), 0.75 (d, 6H, J=6.8 Hz); ^{13}C NMR (100 MHz, $CDCl_3$): 163.88, 163.61, 161.41, 154.82, 146.23, 140.77, 140.67, 138.33, 130.54, 130.45, 121.93, 121.90, 116.05, 115.96, 115.84, 114.06, 113.82, 79.41, 77.36, 77.25, 77.05, 76.73, 44.80, 43.61, 36.57, 33.11, 29.88, 28.74, 28.46, 22.17; EI-MS: 459.6 $[M+H]^+$.

4.2. Biological assays

The cytotoxicity and anti-HBV activities of compounds were evaluated on the HepG2.2.15 cell line. Cytotoxicity was assayed with cell counting kit-8 method (CCK-8; Dojindo, Tokyo, Japan). The anti-HBV antigen secretion activities were assayed by enzyme linked immunosorbent assay (ELISA; Autobio Diagnostics Co., Ltd, China). A real-time PCR assay was used to detect the inhibiting HBV DNA replication of the derivatives[20].

4.2.1. Toxicity measurements[21]

Cytotoxicity induced by the tested compounds to HepG2.2.15 cells was assessed by CCK-8 method. Briefly, HepG2.2.15 cells were cultured in triplicate of 96-well tissue culture plates for 8 days with five different doses of tested compounds. Untreated cells with media alone were used as controls. The CCK-8 solution was added 0.5 h before the end of culture, and then. OD absorbance values at 450 nm were collected by microplate reader (Bio-Rad, model 550) and the cell death percent was calculated[22, 23].

4.2.2. Inhibiting the secretion of HBeAg and HBsAg[24, 25]

Aspirate cell supernatant and detect content of HBeAg and HbsAg by using diagnostic kit (Autobio Diagnostics Co., Ltd, China). The absorbance (A) of the sample was determined on microtiter plate ELISA reader. Drug inhibition ratio = (A normal cell group-A experimental group) / (A blank group-A normal cell group) × 100%. The concentration of compound required for 50% inhibition of HBeAg, HBsAg secretion was defined as IC_{50}.

4.2.3. Real time fluorescent PCR[11]

The supernatants of HepG2.2.15 cells were collected from eight days' culture after the compounds added. And the HBV DNA in the supernatants was quantified by using PCR- fluorescent probing (Quantitative diagnostic kit for HBV DNA). Briefly, 100 μL of the supernatants were added into the extraction buffer, boiled for 10 min and centrifuged for 10 min, and then proper aliquots were used for the fluorescent probing PCR. PCR reaction was run and results were analyzed.

Acknowledgments

The financial support from the National Natural Science Foundation of China (NSFC Nos. 81273354), Key Project of NSFC for International Cooperation (Nos. 81420108027, 30910103908), Major Project of Science and Technology of Shandong Province (2015ZDJS04001), and the Science and Technology Development Project of Shandong Province(No. 2014GSF118175) is gratefully acknowledged.

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Figure captions

Figure 1. Five nucleoside/nucleotide drugs approved by US FDA for HBV treatment

Figure 2. The structures of the anti-HBV natural product Leucamide **A** and it derivatives compound **B**, compound **C** obtained by structural simplification.

Figure 3. The structures of three lead compounds and the newly designed pyrazole series via bioisosterism and pharmacophore hybrid strategy.

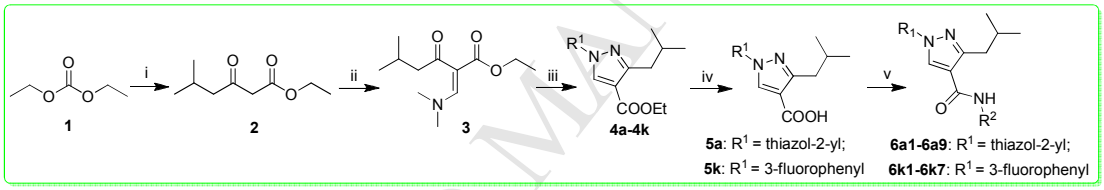
Fig. 4. Inhibitory effect of pyrazole derivatives on HBV DNA level.

Table captions

Table 1. Anti-HBV activity, cytotoxicity and selectivity indices of pyrazole derivatives (**4a-4j**, **6a1-6a9** and **6k1-6k7**) and positive drug lamivudine.

Table 2. Prediction of physicochemical properties[a] of **6a1**, **6a3**, **6a5** and **6k3**.

Schemes



Scheme 1. Reagents and conditions: (i) 4-methylpentan-2-one, NaH, AcOH, THF; (ii) 1,1-dimethoxy-N,N-dimethylmethanamine, 80℃ (iii) EtOH, Et_3N, R^1-$NHNH_2$; (iv) NaOH, H_2O, THF, EtOH; (v) R^2-NH_2, DMF, HATU, Et_3N.

Highlights

1. 26 pyrazole derivatives were prepared.
2. Novel pyrazole derivatives were identified as non-nucleoside HBV inhibitors.
3. Compound **6a3** potently suppressed the secretion of HBsAg and HBeAg.
4. Preliminary SARs of these new derivatives were detailed.