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Design and synthesis of novel benzimidazole derivatives as inhibitors of hepatitis B virus

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ABSTRACT

A series of novel benzimidazole derivatives were synthesized and evaluated for their anti-hepatitis B virus (HBV) activity and cytotoxicity in the HepG2.2.15 cell line. The preliminary SAR was discussed. Compound **12a**, with IC_{50} <0.41 μM and SI >81.2, was the most promising compound and selected as the benchmark compound for further optimization.

1. Introduction

Hepatitis B virus (HBV) infection frequently results in both acute and chronic hepatitis and remains a significant health problem worldwide.[1] Chronic HBV infection will often lead to viral persistence in the absence of a strong antibody or cellular immune response. The number of chronic HBV-infected people is estimated to be more than 400 million worldwide, with about 2 million people carriers of hepatitis B surface antigen (HBsAg). HBV is also associated with a high risk of developing cirrhosis and hepatocellular carcinoma, with roughly 4 million deaths from the resulting cirrhosis and hepatocellular carcinoma every year.[2,3]

The drugs available for the clinical treatment of hepatitis B could be categorized as interferons and nucleotides analogues. Although, α-interferon is effective in treating chronic HBV, the side effects are also serious, such as influenza-like symptoms, depression and insomnia.[4] The nucleoside analogues such as lamivudine (LAM), adefovir (ADV) and entecavir have the advantages of oral administration and fewer side effects (Fig. 1). Some defects (such as exacerbations of hepatitis, limited efficacy and high relapse rate) are still existing.[5–7] To search for new anti-HBV reagents with novel mechanisms, several kinds of non-nucleotide anti-HBV compounds have been investigated recently.[8–14]

Benzimidazole derivatives are of wide interest because of their diverse biological activity and clinical applications.[15–18] Our previous study involving benzimidazole derivative **1a** was screened which exhibited moderate anti-HBV activity. Our previous study involving benzimidazole derivative **1a** indicated that replacement of sulfonyl groups with alkyl substituents in *N*-1 position could dramatically increase anti-viral activities and selectivity (exemplified by compound **1b** and **1c**) (Fig. 2).[19] However, further research indicated that the two compounds had very low water-solubility as well as low oral bioavailability. For these reasons, further studies were initiated in order to find new benzimidazole derivatives with better biological profiles.

In a recent screening, another benzimidazole derivative **2**, which was previously reported by Garuti et al.[20] was identified as an anti-HBV inhibitor with an IC_{50} of 1.37 μM and selectivity index of 131.8. This compound with pyridine moiety exhibited better water-solubility and oral bioavailability than the previous counterparts. Besides, some previous literatures reported that benzimidazole analogues with carboxyl group in position 5 exhibited good antiviral activities.[21–23] Moreover, a recent report suggested *N*-methyl-2-pyrrol in place of 2-pyridyl moiety exhibited good or better antiviral activity.[24]

In the light of the above reports and studies, compound **2** was chosen as the new benchmark compound for further optimization. And our strategies for structure modification were as following (Fig. 3): (1) replacement of sulfonyl groups with alkyl groups in *N*-1 position; (2) introduction of hydrophilic groups into the phenyl ring to probe its effects on the anti-HBV activity; (3) investigation of the different alkyl linker between the benzimidazole moiety and the pyridine; (4) replacement of 2-pyridyl group with *N*-methyl-2-pyrrolly moiety to probe the effect on the anti-HBV activities. In this paper, we report a series of novel benzimidazole derivatives and their anti-HBV activity.

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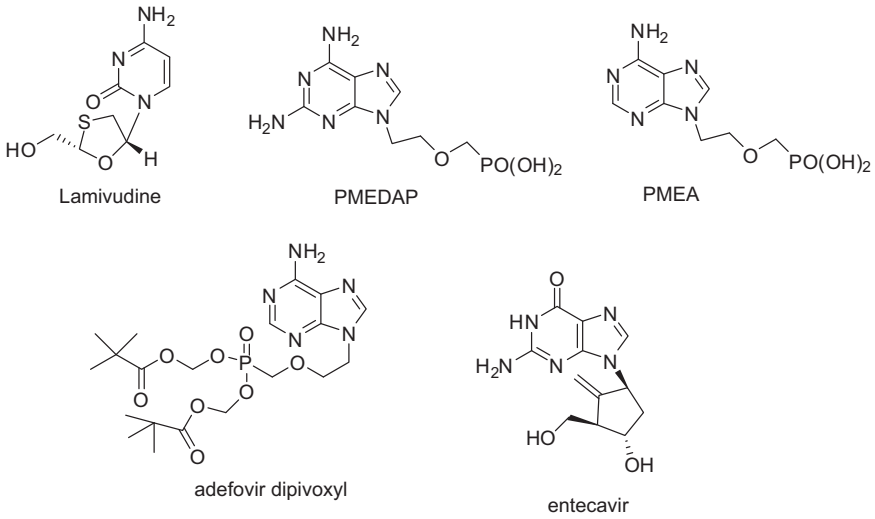


Figure 1. The structures of major antiviral agents for HBV.

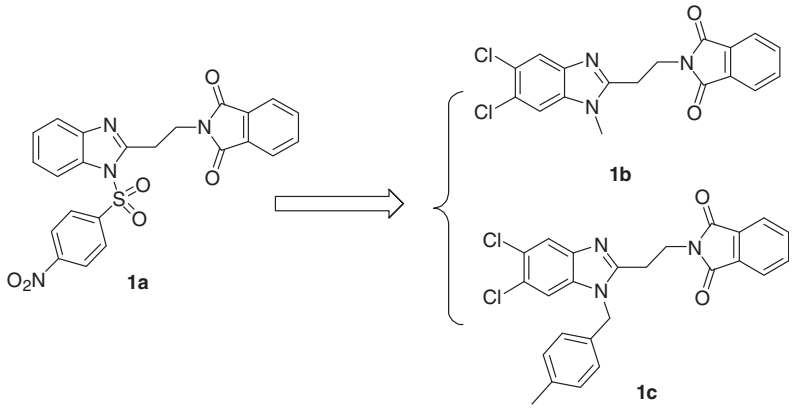


Figure 2. Compounds **1a**, **1b** and **1c**.

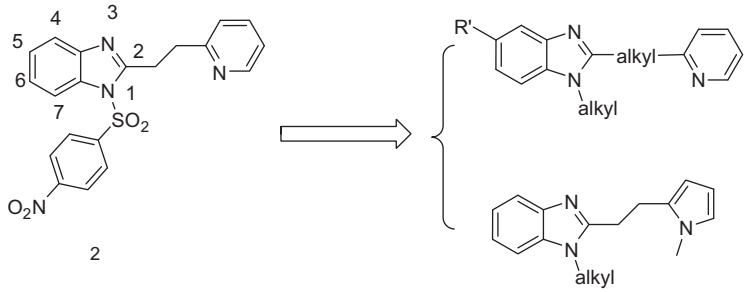


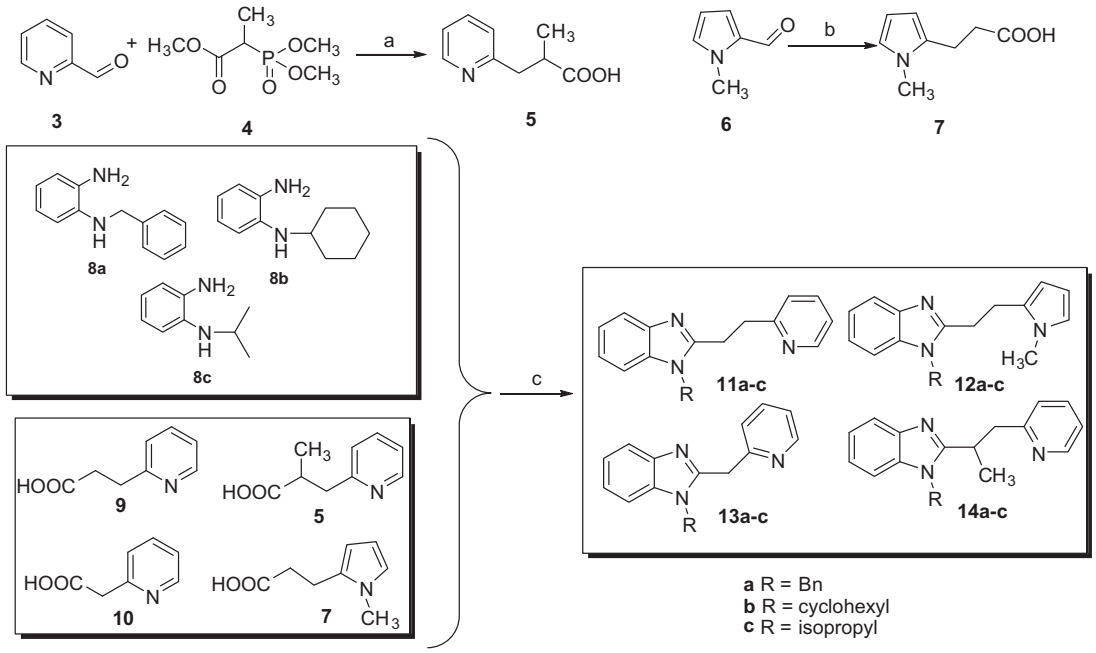
Figure 3.

2. Results and discussion

2.1. Chemistry

The synthesis of one subseries of benzimidazole derivatives were illustrated in Scheme 1. Two acid fragments (compounds **5** and **7**) were first prepared from pyridine-2-aldehyde (**3**) and *N*-methyl-2-pyrrolecarboxaldehyde (**6**), respectively. Thus, pyridine-2-aldehyde (**3**) was subjected to Wittig–Horner reaction, hydrogenation and subsequent hydrolysis to give compound **5** in 81% overall yield. The pyrrol compound **7** was prepared via condensation with propanedioic acid and hydrogenation in an overall yield of 49%. The base fragments **8a–c** were prepared according to the reported methods.[25–27] Thereafter, compound **8a–c** was condensed with different aromatic acids (compounds **5**, **7**, **9** and **10**) using EDAc, and subsequently refluxed in acetic acid to produce the target molecule (Scheme 1).[28,29]

Derivatives with substituents in position 5 was synthesized from intermediates **16a**,**b**, which were prepared from 4-fluoro-3-nitro-benzoic acid methyl ester (**15**) according to reported methods.[30,31] Thus, compounds **16a**,**b** were transformed into benzimidazole compounds **17a**,**b** using abovementioned methods. The



Scheme 1. Reagents and conditions: (a) (i) NaH, THF; (ii) Pd/C, H_2, MeOH; (iii) HCl, reflux; (b) (i) malonic acid, piperidine, pyridine, reflux; (ii) Pd/C, H_2, MeOH; (c) (i) DMAP, EDAc, CH_2Cl_2; (ii) AcOH, reflux.

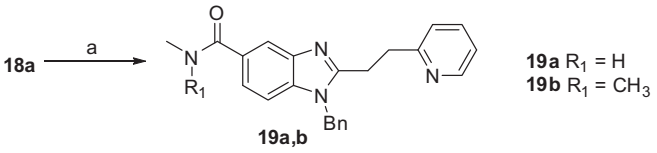
esters **17a**,**b** were hydrolyzed smoothly to give acids **18a**,**b** (Scheme 2). Finally, acid **18a** was condensed with amines to afford two amides **19a**,**b** in good yields (Scheme 3).

2.2. In vitro studies

All the synthesized benzimidazole derivatives were evaluated for their anti-HBV activity and cytotoxicity in HepG 2.2.15 cells, with the antiviral drug lamivudine as reference control.

To identify the effects of the substituents in *N*-1 position as well as replaced 2-pyrrolyl counterparts, two subseries of derivatives **11a**–**c** and **12a**–**c** were synthesized and assessed for anti-HBV activity (Table 1). Compounds **11a**–**c** with different alkyl substituents on position 1 were first prepared and the pharmaceutical profiles were tested. The derivatives **11b** and **11c** exhibited moderate antiviral activities, compared with the reference drug lamivudine, suggesting *N*-1 position might be compatible with a broad range of substituents of different steric characteristics.

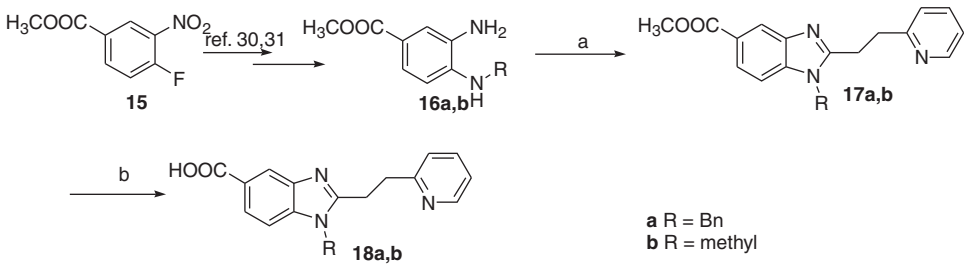
However, it was interesting to note that 2-pyrrolyl compound **12a** (IC_{50} = 0.41 μM, SI = 81.2) showed dramatically increased antiviral activity. Although the anti-HBV activity of compound **12a** (IC_{50} = 0.41 μM) was slightly less potent than the reference control (IC_{50} = 0.16 μM), its cytotoxicity (CC_{50} = 33.3 μM) was over six times less than lamivudine (CC_{50} = 5 μM), resulting in nearly three times higher selectivity than lamivudine.



Scheme 3. Reagents and conditions: (a) amine hydrochloride, DMAP, EDAc, CH_2Cl_2.

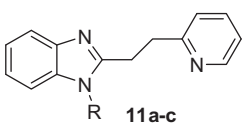
Afterwards, the effects of alkyl linker between the benzimidazole and pyridyl moiety were investigated (Table 2). For compounds **13a**–**c**, the length of the alkyl linker was reduced from ethylene to methylene. Obviously, their anti-HBV activity was dramatically reduced. In addition, compounds **14a**–**c** with one methyl group on the ethylene chain also exhibited low anti-HBV activity. The above results suggested that both length and flexibility of the alkyl linker were important to the antiviral activity. Thus, derivatives with relatively good activities should bear a flexible two-carbon alkyl linker.

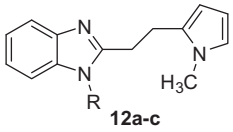
To identify the effects of different polarized substituents on position 5, six benzimidazole compounds (**17**–**19**) were synthesized. However, most of them exhibited poor antiviral activities. The ester substituent (**17a**) with less hydrophilic property indicated much higher antiviral activity than other carboxylic derivatives

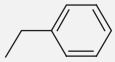
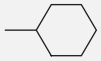
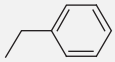
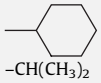


Scheme 2. Reagents and conditions: (a) (i) DMAP, EDAc, CH_2Cl_2; (ii) AcOH, reflux; (b) 6 mol/L HCl, 60 °C.

Table 1
Anti-HBV activity and cytotoxicity of analogues **11a–c** and **12a–c** in vitro

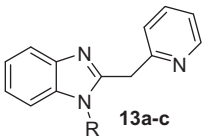


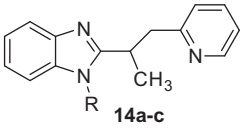


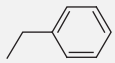
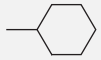
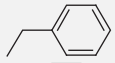
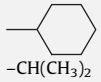
Compound	R	CC_{50}[a] (μM)	IC_{50}[b] (μM)	SI[c]
11a		33.3	NC[d]	—
11b		>100	6.6	>15.2
11c[f]	$-CH(CH_3)_2$	>100	8.6	>11.6
12a		33.3	<0.41	81.2
12b		12.5	>11.1	1.1
12c	$-CH(CH_3)_2$	>100	NA[e]	—
Lamivudine		5	0.16	31.3

'—' The relevant SI cannot be calculated.
[a] Concentrations of compounds required for 50% extinction of HepG 2.2.15 cells.
[b] Concentrations of compounds achieving 50% inhibition of cytoplasmic HBV-DNA synthesis.
[c] Selectivity index (SI) was determined as the CC_{50}/IC_{50} value.
[d] IC_{50} can not be calculated.
[e] Not active.
[f] The oily free base **11c** was converted to its hydrochloride salt to afford a solid.

Table 2
Anti-HBV activity and cytotoxicity of analogues **13a–c** and **14a–c** in vitro





Compound	R	CC_{50}[a] (μM)	IC_{50}[b] (μM)	SI[c]
13a[32]		33.3	NA	—
13b		33.3	>18.2	—
13c	$-CH(CH_3)_2$	>100	NA[d]	—
14a		33.3	NA	—
14b		33.3	NA	—
14c	$-CH(CH_3)_2$	>100	>100	—

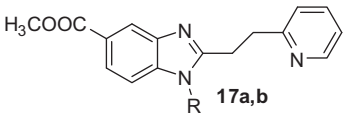
'—' The relevant SI cannot be calculated.
[a] Concentrations of compounds required for 50% extinction of HepG 2.2.15 cells.
[b] Concentrations of compounds achieving 50% inhibition of cytoplasmic HBV-DNA synthesis.
[c] Selectivity index (SI) was determined as the CC_{50}/IC_{50} value.
[d] Not active.

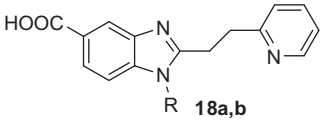
(**18a**,**b** and **19a**,**b**), suggesting that lipophilic property was an important determinant for the anti-HBV activity (Table 3).

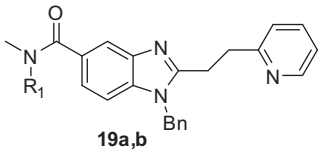
3. Conclusion

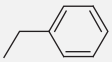
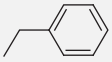
In summary, a series of novel benzimidazole analogues were prepared and assessed for their anti-HBV activity and cytotoxicity in vitro. The preliminary SAR was discussed. Compounds **12a** and **17a** showed potent antiviral activities (IC_{50} <0.41 μM and IC_{50} = 2.1 μM, respectively) and high selectivity (SI >81.2 and SI = 47.6, respectively). Further studies of SAR based on **12a** are going on in our group.

Table 3
Anti-HBV Activity and Cytotoxicity of Analogues **17a**,**b**, **18a**,**b** and **19a**,**b** in vitro







Compound	R	R_1	CC_{50}[a] (μM)	IC_{50}[b] (μM)	SI[c]
17a		—	>100	2.1	>47.6
17b	$-CH_3$	—	>100	NA[d]	—
18a		—	>100	>100	—
18b	$-CH_3$	—	>100	NA	—
19a	—	H	>100	NA	—
19b	—	$-CH_3$	>100	>100	

'—' The relevant SI cannot be calculate.
[a] Concentrations of compounds required for 50% extinction of HepG 2.2.15 cells.
[b] Concentrations of compounds achieving 50% inhibition of cytoplasmic HBV-DNA synthesis.
[c] Selectivity index (SI) was determined as the CC_{50}/IC_{50} value.
[d] Not active.

4. Materials and methods

4.1. General

^1H NMR spectral data were recorded in DMSO-d_6, $CDCl_3$ and D_2O on Varian Mercury 500 NMR spectrometer and ^{13}C NMR data were recorded in DMSO-d_6, $CDCl_3$ and D_2O on Varian Mercury 100 NMR spectrometer. Chemical shifts (δ) are reported in parts per million (ppm), and the signals are described as s (singlet), d (doublet), t (triplet), q (quarter), m (multiple), and dd (doublet of doublet). Coupling constants (J values) are given in Hz. Low-resolution mass spectra (MS) and high-resolution mass spectra (HRMS) were recorded at an ionizing voltage of 70 eV on a Agilent/5973 N spectrometer and Waters Micromass GCT. Column chromatography was carried out on silica gel (300 mesh). Compounds **9** and **10** were purchased from Aldrich. All reactions were monitored using thin-layer chromatography (TLC) on silica gel plates.

4.2. Synthesis

4.2.1. Preparation of 2-methyl-3-(pyridin-2-yl)propanoic acid (5)

To a suspension of NaH (7 g, 0.17 mol) in dry THF at 0 °C was added dropwise methyl 2-(dimethoxyphosphoryl)propanoate (13 g, 0.06 mol) and stirred for 1 h. Then picolinaldehyde (5.5 mL, 0.06 mol) was dropped into the mixture and stirred at room temperature for 6 h. After the reaction was completed, the mixture was poured into water (100 mL) and extracted with dichloromethane (10 mL × 3). The organic layer was separated and washed with saturated brine (10 mL × 2), and dried over Na_2SO_4 and evaporated

to dryness to give a residue, which was hydrogenated (1 atm.) on 10% Pd/C (0.8 g) in methanol for 7 h. The Pd/C was filtrated off and the organic phase was concentrated under reduced pressure to give a pale yellow residue. The residue was suspended in 6 mol/L HCl (40 mL) and heated at 60 °C for 5 h. The mixture was treated with aqueous 6 mol/L NaOH (40 mL). The resulting solution was extracted with ethyl acetate (10 mL × 3). The organic portions were combined, dried ($MgSO_4$) and filtered. The solution was concentrated to give compound **5** (8.1 g, 81%) as a white solid, mp 100–101 °C. ^{1}H NMR (DMSO-d_6): δ 8.43–8.45 (d, J = 4.5 Hz, 1H), 7.54–7.57 (m, 1H), 6.99–7.15 (m, 2H), 3.15–3.19 (m, 1H), 3.01–3.05 (m, 1H), 2.79–2.83 (m, 1H), 1.02 (d, J = 7 Hz, 3H). LRMS (EI): m/z 137 [M]$^+$. HRMS (EI): calcd for $C_{29}H_{11}NO_2$ 165.0790, found 165.0789.

4.2.2. Preparation of 3-(1-methyl-1*H*-pyrrol-2-yl)propanoic acid (7)

A mixture of malonic acid (20 g, 0.2 mol), 1-methyl-1*H*-pyrrole-2-carbaldehyde (10.8 g, 0.1 mol) and piperidine (1.2 mL, 0.01 mol) in pyridine (30 mL) was heated under N_2 at 90 °C for 3 h then cooled to room temperature and poured into water. The resulting aqueous mixture was neutralized with 2 mol/L HCl (25 mL). The resulting precipitate was collected by filtration to give yellow solid (7.5 g, 50%). The yellow solid (7.5 g, 0.05 mol) was hydrogenated (1 atm.) on 10% Pd/C (0.7 g) in methanol for 2 h at room temperature. Then Pd/C was filtrated off and the organic phase was concentrated to dryness to give 7.4 g of compound **7** as a yellow solid (49% yield), mp 87–89 °C (lit.[33] mp 80–82 °C). ^{1}H NMR ($CDCl_3$): δ 6.57–6.58 (t, 1H), 6.06–6.07 (t, 1H), 5.91–5.92 (m, 1H), 3.56 (s, 3H), 2.88 (t, J = 15.6 Hz, 2H), 2.72 (t, J = 15.6 Hz, 2H). LRMS (EI): m/z 153 [M]$^+$.

4.2.3. General procedure for the preparation of compounds 11a–c

A mixture of compounds **8a**–**c** (0.70 mmol) and **9** (1.5 mmol) was stirred overnight in dry CH_2Cl_2 at room temperature, with 4-(dimethylamino) pyridine (0.15 mmol) as the catalyst and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (2.2 mmol) as condensation agent. The resulting solution was poured into saturated NH_4Cl. Then it partitioned between chloroform and water. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuum.

The immediate product was directly poured into dry acetic acid and was refluxed for about 5 h. The mixture was neutralized to pH 7 with saturated Na_2CO_3. The precipitate was filtered, washed with water, and dried to give the compound **11a**–**c**.

4.2.3.1. 1-Benzyl-2-[2-(pyridin-2-yl)-ethyl]-1*H*-benzoimidazole (11a). The crude product was recrystallized from ethyl acetate and petroleum ether to afford a yellow solid (95 mg, 40% yield), mp 87–88 °C. ^{1}H NMR ($CDCl_3$): δ 8.50 (d, J = 5 Hz, 1H), 7.76 (d, J = 8 Hz, 1H), 7.54 (d, J = 2 Hz, 1H), 7.22–7.26 (m, 4H), 7.16–7.18 (m, 3H), 7.09–7.11 (m, 1H), 6.99–7.01 (m, 1H), 5.32 (s, 2H), 3.43 (t, J = 15 Hz, 2H), 3.33(t, J = 15 Hz, 2H). ^{13}C NMR ($CDCl_3$): δ 160.09, 154.63, 149.20 142.68, 136.35, 135.92, 135.29, 128.83, 127.68, 126.12, 123.28, 122.22, 121.91, 121.37, 119.19, 109.55, 46.70, 35.54, 26.75. LRMS (EI): m/z 313.2 [M]$^+$. HRMS (EI): calcd for $C_{21}H_{19}N_3$ 313.1579, found 313.1580.

4.2.3.2. 1-Cyclohexyl-2-[2-(pyridin-2-yl)-ethyl]-1*H*-benzoimidazole (11b). The crude product was recrystallized from ethyl acetate and petroleum ether to afford a yellow solid (79 mg, 34% yield), mp 120–121 °C. ^{1}H NMR ($CDCl_3$): δ 8.57 (d, J = 4 Hz, 1H), 7.73–7.74 (t, J = 8.5 Hz, 1H), 7.56–7.72 (m, 1H), 7.51–7.53 (m, 1H), 7.13–7.20 (m, 4H), 4.24–4.26 (m, 1H), 3.41–3.45 (m, 2H), 3.35–3.39 (m, 2H), 2.17–2.22 (m, 2H), 1.91–1.94 (m, 2H), 1.74–1.80 (m, 3H), 1.39–1.45 (m, 2H), 1.29–1.39 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 160.31, 153.86, 149.27, 143.22, 136.46, 133.65, 123.36, 121.39, 121.37, 121.21, 119.34, 111.87, 55.88, 36.05, 31.16, 27.41, 25.97, 25.27. LRMS (EI): m/z 305.1 [M]$^+$. HRMS (EI): calcd for $C_{20}H_{23}N_3$ 305.1892, found 305.1890.

4.2.3.3. 1-Isopropyl-2-[2-(pyridin-2-yl)-ethyl]-1*H*-benzoimidazole (11c). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 3:1) to afford light a yellow oily free base (60 mg, 30% yield). ^{1}H NMR (DMSO-d_6): δ 8.71 (d, J = 4.5 Hz, 1H), 8.25–8.26 (m, 1H), 8.13 (d, J = 7.5 Hz, 1H), 7.80–7.83 (t, J = 15.5 Hz, 2H), 7.69–7.70 (m, 1H), 7.53–7.57 (m, 2H), 5.14–5.19 (m, 1H), 3.75–3.78 (t, J = 15 Hz, 2H), 3.57–3.60 (t, J = 15 Hz, 2H), 1.65 (d, J = 7 Hz, 6 H). LRMS (EI): m/z 265 [M]$^+$.

A solution of the oily free base in CH_2Cl_2 was purged into the hydrochloride gas for 0.5 h, following it was deposited and filtrated to afford a light a yellow solid (69 mg), mp 63–64 °C. ^{1}H NMR (D_2O): δ 8.64–8.65 (m, 1H), 8.39 (d, J = 4 Hz, 1H), 7.96 (d, J = 3.5 Hz, 1H), 7.84–7.85 (m, 2H), 7.68–7.69 (m, 1H), 7.53 (d, J = 9.5 Hz, 2H), 4.97–5.00 (m, 1H), 3.70–3.73 (m, 2H), 3.58–3.60 (m, 2H), 1.65 (d, J = 6.5 Hz, 6H). ^{13}C NMR (D_2O): δ 153.40, 150.01, 148.20, 142.52, 131.29, 130.83, 127.95, 127.18, 126.69, 126.66, 115.42, 114.95, 51.90, 30.86, 25.26, 20.84. HRMS (EI): calcd for $C_{17}H_{20}N_3Cl$ 301.1346, found 301.1360.

4.2.4. General procedure for the preparation of compounds 12a–c

Compounds **12a**–**c** were synthesized from compounds **8a**–**c** and **7** using the procedure for the preparation of **11a**–**c**.

4.2.4.1. 1-Benzyl-2-(2-(1-methyl-1*H*-pyrrol-2-yl)ethyl)-1*H*-benzoimidazole (12a). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 1:2) to afford a white solid (143 mg, 60% yield), mp 97–98 °C. ^{1}H NMR ($CDCl_3$): δ 7.79 (d, J = 7.5 Hz, 1H), 7.22–7.30 (m, 6H), 7.01 (d, J = 6.5 Hz, 2H), 6.53–6.54 (t, J = 4 Hz, 1H), 6.04–6.05 (t, J = 6 Hz, 1H), 5.88–8.89 (t, J = 3.5 Hz, 1H), 5.23 (s, 2H), 3.43 (s, 3H), 3.13 (s, 4H). ^{13}C NMR ($CDCl_3$): δ 154.39, 142.60, 135.95, 131.81, 129.01, 127.89, 126.11, 122.49, 122.12, 121.54, 119.33, 109.45, 106.76, 105.72, 46.59, 33.51, 27.45, 24.24. MS (ESI): m/z 316.2 [M+1]$^+$. HRMS (ESI): calcd for $C_{21}H_{22}N_3H$ [M+H]$^+$ 316.1812, found 316.1808.

4.2.4.2. 1-Cyclohexyl-2-[2-(1-methyl-1*H*-pyrrol-2-yl)ethyl]-1*H*-benzoimidazole (12b). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 1:2.5) to afford a yellow solid (145 mg, 62.5% yield), mp 67–68 °C. ^{1}H NMR ($CDCl_3$): δ 7.73–7.75 (m, 1H), 7.52–7.54 (m, 1H), 7.18–7.21 (m, 2H), 6.55–6.56 (t, J = 4 Hz, 1H), 6.08–6.09 (t, J = 6 Hz, 1H), 6.00–6.01 (t, J = 3 Hz, 1H), 4.12–4.16 (m, 1H), 3.53 (s, 3H), 3.17–3.20 (m, 4H), 2.21–2.24 (m, 2H), 1.94–2.04 (m, 2H), 1.79–1.85 (m, 3H), 1.41–1.43 (m, 2H), 1.25–1.38 (m, 1H). ^{13}C NMR ($CDCl_3$): δ 153.50, 143.02, 133.66, 131.89, 121.59, 121.52, 121.38, 119.37, 111.81, 106.77, 105.70, 105.55, 56.25, 33.61, 31.25, 30.94, 28.14, 26.07, 25.26, 24.57. LRMS (EI): m/z 307 [M]$^+$. HRMS (EI): calcd for $C_{20}H_{25}N_3$ 307.2049, found 307.2048.

4.2.4.3. 1-Isopropyl-2-(2-(1-methyl-1*H*-pyrrol-2-yl)ethyl)-1*H*-benzoimidazole (12c). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 1:1) to afford a yellow solid (94 mg, 46.5% yield), mp 42–43 °C. ^{1}H NMR ($CDCl_3$): δ 7.74–7.76 (m, 1H), 7.50–7.51 (m, 1H), 7.20–7.22 (m, 2H), 6.56–6.57 (t, J = 4 Hz, 1H), 6.08–6.09 (t, J = 6 Hz, 1H), 5.99–6.00 (m, 1H), 4.64–4.69 (m, 1H), 3.54 (s, 3H), 3.17–3.21 (m, 4H), 1.60 (d, J = 7 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 153.36, 143.17, 133.35, 131.89, 121.67, 121.53, 121.44, 119.46, 111.43, 106.74, 105.53, 60.33, 47.73, 33.63, 27.83, 24.47, 21.33. LRMS (EI): m/z 267 [M]$^+$. HRMS (EI): calcd for $C_{17}H_{21}N_3$ 267.1735, found 267.1738.

4.2.5. General procedure for the preparation of compounds 13a–c

Compounds **13a**–**c** were synthesized from compounds **8a**–**c** and **10** using the procedure for the preparation of **11a**–**c**.

4.2.5.1. 1-Benzyl-2-(pyridin-2-yl-methyl)-1*H*-benzoimidazole (13a). The crude product can be used directly without further purification as a yellow solid (91 mg, 40% yield), mp 137–138 °C. ^{1}H NMR (DMSO-d_6): δ 8.48–8.49 (m, 1H), 7.85–7.93 (m, 1H), 7.72–7.84 (m, 1H), 7.67–7.70 (m, 2H), 7.49–7.57 (m, 2H), 7.31–7.41 (m, 1H), 7.18–7.31 (m, 5H), 5.89 (s, 2H), 5.03 (s, 2H). ^{13}C NMR (DMSO-d_6): δ 150.72, 147.25, 147.02, 134.12, 131.99, 130.95, 128.70, 128.62, 128.04, 127.10, 126.82, 126.07, 125.73, 125.38, 123.94, 114.62, 113.16, 47.94, 32.02. LRMS (EI): *m/z* 299 [M]$^+$. HRMS (EI): calcd for $C_{20}H_{17}N_3$ 299.1422, found 299.1426.

4.2.5.2. 1-Cyclohexyl-2-(pyridin-2-yl-methyl)-1*H*-benzoimidazole (13b). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 1:1) to afford **13b** as a light yellow solid (86 mg, 39% yield), mp: 180–181 °C. ^{1}H NMR (DMSO-d_6): δ 8.49–8.50 (m, 1H), 8.17–8.19 (m, 1H), 7.94–7.97 (m, 1H), 7.84–7.85 (m, 1H), 7.71–7.73 (m, 1H), 7.53–7.59 (m, 2H), 7.40–7.42 (m, 1H), 5.00 (s, 2H), 4.58 (m, 1H), 2.14–2.21 (m, 2H), 1.78–1.80 (m, 2H), 1.70–1.73 (m, 2H), 1.61–1.63 (m, 1H), 1.28–1.41 (m, 3H). ^{13}C NMR (DMSO-d_6): δ 152.73, 150.09, 148.02, 139.35, 130.73, 130.08, 125.77, 125.40, 124.72, 123.63, 115.03, 114.48, 57.71, 32.85, 29.58, 24.94, 24.01. LRMS (EI): *m/z* 291 [M]$^+$. HRMS (EI): calcd for $C_{19}H_{21}N_3$ 291.1735, found 291.1736.

4.2.5.3. 1-Isopropyl-2-(pyridin-2-yl-methyl)-1*H*-benzoimidazole (13c). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 1:2) to afford **13c** as a light yellow solid (78 mg, 41% yield), mp 139–140 °C. ^{1}H NMR ($CDCl_3$): δ 8.53 (d, J = 4 Hz, 1H), 7.75–7.77 (m, 1H), 7.57–7.58 (m, 1H), 7.49–7.50 (m, 1H), 7.15–7.26 (m, 4H), 4.86–4.92 (m, 1H), 4.53 (s, 2H), 1.45 (d, J = 7 Hz, 6H). ^{13}C NMR ($CDCl_3$): δ 156.77, 151.42, 149.05, 143.10, 136.71, 133.28, 123.22, 121.84, 121.79, 121.44, 119.60, 111.69, 48.16, 37.80, 20.77. LRMS (EI): *m/z* 251 [M]$^+$. HRMS (EI): calcd for $C_{16}H_{17}N_3$ 251.1422, found 251.1423.

4.2.6. General procedure for the preparation of compounds 14a–c

Compounds **14a**–**c** were synthesized from compounds **8a**–**c** and **5** using the procedure for the preparation of **11a**–**c**.

4.2.6.1. 1-Benzyl-2-[1-(pyridin-2-yl)propan-2-yl]-1*H*-benzoimidazole (14a). The crude product can be used directly without further purification as a yellow solid (101 mg, 41% yield), mp 131–132 °C. ^{1}H NMR (DMSO-d_6): δ 8.60 (d, J = 5 Hz, 1H), 8.20–8.21 (d, J = 7 Hz, 1H), 7.81–7.83 (m, 2H), 7.72–7.74 (m, 1H), 7.67–7.68 (m, 1H), 7.49–7.55 (m, 2H), 7.19–7.31 (m, 3H), 7.16–7.18 (m, 2H), 5.80–5.92 (m, 2H), 4.31–4.35 (m, 1H), 3.81–3.86 (m, 1H), 3.53–3.60 (m, 1H), 1.32 (d, J = 7 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ 155.75, 153.01, 144.20, 143.45, 134.65, 131.89, 131.12, 128.95, 128.16, 126.96, 126.86, 126.14, 125.79, 124.90, 114.48, 113.18, 47.68, 37.67, 30.76, 18.48. LRMS (EI): *m/z* 327 [M]$^+$. HRMS (EI): calcd for $C_{22}H_{21}N_3$ 327.1735, found 327.1731.

4.2.6.2. 1-Cyclohexyl-2-[1-(pyridin-2-yl)propan-2-yl]-1*H*-benzoimidazole (14b). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 1:1) to afford **14b** as a yellow solid (104 mg, 43% yield), mp 153–154 °C. ^{1}H NMR (DMSO-d_6): δ 8.65–8.66 (m, 1H), 8.21–8.22 (m, 1H), 8.12 (d, J = 8 Hz, 1H), 7.80 (d, J = 8 Hz, 1H), 7.75–7.76 (m, 1H), 7.66–7.67 (m, 1H), 7.47–7.54 (m, 2H), 4.74–4.79 (m, 1H), 4.49–4.50 (m, 1H), 3.74–3.78 (m, 1H), 3.55–3.59 (m, 1H), 2.14–2.20 (m, 2H), 1.92–1.95 (m, 1H), 1.76–1.83 (m, 2H), 1.64–1.66 (m, 1H), 1.53–1.57 (m, 3H), 1.46–1.47 (m, 3H), 1.40–1.42 (m, 1H). ^{13}C NMR (DMSO-d_6): δ 154.86, 153.33, 144.06, 143.52, 131.22, 129.90, 127.02, 125.73, 125.24, 124.84, 115.17, 114.39, 57.35, 38.09, 30.69, 30.08, 29.92, 24.84, 24.80, 24.06, 18.70. LRMS (EI): *m/z* 319 [M]$^+$. HRMS (EI): calcd for $C_{21}H_{25}N_3$ 319.2050, found 319.2048.

4.2.6.3. 1-Isopropyl-2-[1-(pyridin-2-yl)propan-2-yl]-1*H*-benzoimidazole (14c). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 1:1) to afford **14c** as a red solid (85 mg, 40% yield), mp 140–141 °C. ^{1}H NMR (DMSO-d_6): δ 8.66 (d, J = 4.5 Hz, 1H), 8.22–8.23 (m, 1H), 8.12 (d, J = 8 Hz, 1H), 7.94 (d, J = 7.5 Hz, 1H), 7.76 (d, J = 7.5 Hz, 1H), 7.67–7.68 (m, 1H), 7.52–7.58 (m, 2H), 5.21–5.26 (m, 1H), 4.34–4.40 (m, 1H), 3.76–3.81 (m, 1H), 3.54–3.59 (m, 1H), 1.64 (d, J = 7 Hz, 3H), 1.55–1.56 (d, J = 7 Hz, 3H), 1.49–1.50 (d, J = 7 Hz, 3H). ^{13}C NMR (DMSO-d_6): δ 154.62, 153.34, 144.07, 143.64, 131.21, 129.66, 127.05, 125.81, 125.37, 124.88, 114.81, 114.45, 50.12, 30.78, 20.53, 20.43, 18.44. LRMS (EI): *m/z* 279 [M]$^+$. HRMS (EI): calcd for $C_{18}H_{21}N_3$ 279.1735, found 279.1732.

4.2.7. General procedure for the preparation of compounds 17a and 17b

Compounds **17a** and **17b** were synthesized from compounds **16a**,**b**, respectively, and compound **9** using the procedure for the preparation of **11a**–**d**.

4.2.7.1. Methyl 1-benzyl-2-(2-(pyridin-2-yl)ethyl)-1*H*-benzoimidazole-5-carboxylate (17a). The crude product was purified through a silica gel column (ethyl acetate/petroleum ether = 3:1) to afford **17a** as a pale yellow solid (118 mg, 42% yield), mp 122–123 °C. ^{1}H NMR ($CDCl_3$): δ 8.47–8.50 (m, 1H), 7.91–7.93 (m, 1H), 7.53–7.55 (m, 1H), 7.26–7.27 (m, 3H), 7.17–7.21 (m, 2H), 7.09–7.11 (m, 1H), 6.98–7.00 (m, 2H), 5.34 (s, 2H), 3.93 (s, 3H), 3.40–3.44 (t, J = 18.7 Hz, 2H), 3.33–3.36 (t, J = 18.7 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 167.66, 159.87, 156.59, 149.25, 142.33, 138.63, 136.45, 135.45, 129.01, 127.97, 126.15, 124.26, 124.03, 123.40, 121.57, 121.51, 109.26, 52.01, 46.99, 35.32, 26.76. LRMS (EI): *m/z* 371 [M]$^+$. HRMS (EI): calcd for $C_{23}H_{21}N_3O_2$ 371.1634, found 371.1635.

4.2.7.2. Methyl 1-methyl-2-[2-(pyridin-2-yl)ethyl]-1*H*-benzoimidazole-5-carboxylate (17b). The crude product was purified through a silica gel column (ethyl acetate/methanol = 30:1) to afford **17b** as a yellow solid (100 mg, 45% yield), mp 111–112 °C. ^{1}H NMR ($CDCl_3$): δ 8.55 (d, J = 5 Hz, 1H), 8.44–8.45 (m, 1H), 7.97–7.99 (m, 1H), 7.57–7.59 (m, 1H), 7.277.29 (m, 1H), 7.16–7.20 (m, 1H), 7.14–7.15 (m, 1H), 3.94 (s, 3H), 3.68 (s, 3H), 3.37–3.43 (m, 4H). ^{13}C NMR ($CDCl_3$): δ 174.02, 167.67, 159.70, 156.39, 149.05, 141.80, 138.77, 136.74, 124.04, 123.79, 123.44, 121.65, 121.22, 108.66, 51.98, 35.35, 29.83, 26.79. LRMS (EI): *m/z* 295 [M]$^+$. HRMS (EI): calcd for $C_{17}H_{17}N_3O_2$ 295.1321, found 295.1319.

4.2.8. General procedure for the preparation of compounds 18a and 18b

Compound **17a** or **17b** (0.32 mmol) was suspended in 6 mol/L HCl (5 mL) and heated at 60 °C for 5 h. The mixture was treated with aqueous 6 mol/L NaOH (5 mL). The formed precipitate was filtered, washed with water, and dried to give the target compound.

4.2.8.1. 1-Benzyl-2-[2-(pyridin-2-yl)ethyl]-1*H*-benzoimidazole-5-carboxylic acid (18a). The crude product can be used directly without further purification as a white solid (94 mg, 83% yield), mp 198–199 °C. ^{1}H NMR (DMSO-d_6): δ 8.46–8.47 (m, 1H), 8.16–8.17 (m, 1H), 7.79 (d, J = 7.5 Hz, 1H), 7.67 (s, 1H), 7.53 (d, J = 7.5 Hz, 1H), 7.26–7.30 (m, 4H), 7.19–7.20 (m, 1H), 7.08–7.09 (d, J = 5.5 Hz, 2H), 5.50 (s, 2H), 3.29–3.31 (m, 4H). ^{13}C NMR

(DMSO-d_6): δ 171.64, 159.87, 156.69, 148.89, 141.83, 140.56, 140.16, 138.55, 136.61, 136.40, 128.77, 127.52, 127.34, 126.44, 123.23, 122.98, 121.45, 120.21, 109.95, 46.14, 34.35, 26.02. LRMS (EI): *m/z* 357 $[M]^+$. HRMS (EI): calcd for $C_{22}H_{19}N_3O_2$ 357.1477, found 357.1478.

4.2.8.2. 1-Methyl-2-[2-(pyridin-2-yl)ethyl]-1*H*-benzoimidazole-5-carboxylic acid (18b). The crude product can be used directly without further purification as a yellow solid (89 mg, 99% yield), mp 220–221 °C. ^{1}H NMR (DMSO-d_6): δ 8.70–8.71 (d, J = 4.5 Hz, 1H), 8.29–8.30 (m, 1H), 8.24–8.25 (d, J = 1Hz, 1H), 8.03–8.05 (m, 1H), 7.90–7.94 (m, 2H), 7.72 (d, J = 6 Hz, 1H), 3.99 (s, 3H), 3.66–3.70 (m, 4H). ^{13}C NMR (DMSO-d_6): δ 166.66, 154.70, 144.28, 142.95, 135.97, 132.18, 127.57, 126.61, 125.57, 124.72, 116.20, 112.39, 100.08, 31.35, 29.93, 24.76. LRMS (EI): *m/z* 281 $[M]^+$. HRMS (EI): calcd for $C_{16}H_{15}N_3O_2$ 281.1164, found 281.1167.

4.2.9. General procedure for the preparation of compounds 19a and 19b

To a solution of compound **18a** (0.32 mmol) in dry CH_2Cl_2 was added methylamine hydrochloride or dimethylamine hydrochloride (0.6 mmol), with 4-(dimethylamino) pyridine (0.06 mmol) as the catalyst and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (0.96 mmol) as condensation agent. The resulting solution was stirred overnight at room temperature and poured into saturated NH_4Cl. Then it partitioned between chloroform and water. The organic layer was washed with water and brine, dried over Na_2SO_4, and concentrated in vacuum.

4.2.9.1. 1-Benzyl-*N*-methyl-2-(2-(pyridin- 2-yl)ethyl)-1*H*-benzoimidazole-5-carboxamide (19a). The crude product was purified through a silica gel column (ethyl acetate/methanol = 10:1) to afford **19a** as a pale yellow solid (107 mg, 91.3% yield), mp 83–84 °C. ^{1}H NMR ($CDCl_3$): δ 8.46–8.47 (m, 1H), 8.10–8.11 (m, 1H), 7.67–7.69 (m, 1H), 7.49–7.52 (m, 1H), 7.22–7.25 (m, 3H), 7.96–7.16 (m, 3H), 6.94–6.95 (m, 2H), 6.47–6,48 (m, 1H), 5.29 (s, 2H), 3.31–3.38 (m, 4H), 2.98 (d, J = 4.5 Hz, 3H). ^{13}C NMR ($CDCl_3$): δ 168.72, 159.85, 149.23, 142.29, 136.45, 135.49, 128.98, 127.94, 126.14, 123.37, 121.96, 121.53, 117.71, 109.66, 94.93, 46.98, 35.36, 26.91, 26.75. LRMS (EI): *m/z* 370 $[M]^+$. HRMS (EI): calcd for $C_{23}H_{22}N_4O$ 370.1794, found 370.1801.

4.2.9.2. 1-Benzyl-*N*,*N*-dimethyl-2-(2-(pyridin-2-yl)ethyl)-1*H*-benzoimidazole-5-carboxamide (19b). The crude product was purified through a silica gel column (ethyl acetate/methanol = 20:1) to afford **19b** as a white solid (114 mg, 93% yield), mp 92–93 °C. ^{1}H NMR ($CDCl_3$): δ 8.50 (d, J = 4 Hz, 1H), 7.80–7.81 (m, 1H), 7.54–7.55 (m, 1H), 7.29–7.31 (m, 1H), 7.24–7.26 (m, 4H), 7.20 (s, 1H), 7.17–7.18 (m, 1H), 7.15–7.16 (m, 1H), 6.97–6.99 (m, 2H), 5.32 (s, 2H), 3.42 (t, J = 15 Hz, 2H), 3.34 (t, J = 15 Hz, 2H), 3.12 (d, J = 35 Hz, 3H), 3.05 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 172.12, 159.94, 156.02, 149.23, 142.03, 136.42, 136.13, 135.62, 130.18, 128.96, 127.89, 126.17, 123.33, 122.08, 121.46, 118.34, 109.72, 46.93, 35.47, 26.77. LRMS (EI): *m/z* 384 $[M]^+$. HRMS (EI): calcd for $C_{24}H_{24}N_4O$ 384.1950, found 384.1953.

4.3. Biological assays

4.3.1. Cell culture and antiviral assays

Details of the design of the antiviral procedure and the growth conditions for HepG2.2.15 cells have been previously described.[34–36] Briefly, confluent cultures in 96-well tissue culture plates were treated with various doses of antiviral compounds in minimal essential medium (MEM) supplemented with 10% fetal bovine serum. Fresh MEM with the same concentration of compounds was replaced at day 4, and the supernatants were harvested at day 8, then the extracellular (virion) HBV-DNA were measured by real time fluorescent PCR.

4.3.2. Toxicity measurements

Cytotoxicity of compounds was assessed by the MTT assay as previously described.[34–36] Briefly, HepG2.2.15 cells were cultured in triplicate of 96-well tissue culture plates for 8 days with various doses of tested compounds. The cells with media alone were used as controls. MTT (5 g/L) reagent was added 4 h before the end of culture, and then cells were lysed with 10% sodium dodecyl sulfate (SDS), 50% DMF, pH 7.2. O.D. values were read at 570 nm 6 h later and the cell death percent was calculated.

4.3.3. Real time fluorescent PCR

The supernatants of HepG2.2.15 cells were collected from 8 days culture after the compounds added. The HBV-DNA in the supernatants was quantified by using fluorescent PCR. Briefly, 50 μL of the supernatants were added into the extraction buffer, boiled for 10 min and centrifuged for 5 min, and then proper aliquots were used for the fluorescent PCR. PCR primers were:

P1: 5′-ATCCTGCTGCTATGCCTCATCTT-3′,

P2: 5′-ACAGTGGGGAAAGCCCTACGAA-3′.

The probe was 5′-TGGCTAGTTTACTAGTGCCATTTTG-3′. PCR reaction was run at MJ Research PTC-200, and results were analyzed by software OpticonMonitor v2.01.

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