

Design and synthesis of a novel 5-(aminomethylene)thiazolidine-2,4-dione derivatives as potent hepatitis-B virus polymerase inhibitors

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Abstract

A series of novel thiazolidinedione analogues (TZD) were designed and synthesized potent inhibitors of HBV capsid assembly. The synthesis of thiazolidine-2,4-dione derivatives (**4a-4o**), starting from the condensation of 5-(ethoxymethylene)thiazolidine-2,4-dione (**1**) with various secondary amines (**3**) derived from biologically active compounds. The newly synthesized TZD analogues **4a-4o** were characterized by ¹H NMR, ¹³C NMR, and MS and evaluated for their anti-HBV activity. Most of the compounds inhibited the expression of viral antigens at low concentration. Six compounds, **4g**, **4h**, **4l**, **4m**, **4n**, and **4o**, demonstrated potent inhibition of HBV DNA replication at the submicromolar range. Of these five initial hits, compound **4o** was the most active when compared with lamivudine.

Introduction

Worldwide more than two billion people were seriously affected by hepatitis B virus (HBV), which can cause Liver cirrhosis and hepatocellular carcinoma in humans. It was estimated that 400 million people were chronically infected with HBV worldwide up to recent times, and HBV was responsible for 750,000 deaths each year (Custer et al., 2004). HBV infections in developing countries increase abnormally. The current therapies including vaccines, immunomodulators, interferon- α , polyethylene glycol interferon- α and nucleoside drugs for treating HBV are still unsatisfactory, due to high recurrence, drug resistance and inevitable side effects including influenza-like illness, myalgia, headache, reduction of neutrophilic granulocyte and blood platelet, etc (Sato et al., 2010; Locarnini et al., 2006; Wong et al, 1993; Fattovich et al, 1988). Therefore, it is interesting to explore novel classes of drugs with different antiviral targets and mechanisms for anti-HBV purposes.

On the other hand, the thiazolidinedione analogues were investigated for the treatment of a variety of

diseases, e.g., as anti-cancer (Havrylyuk et al., 2009), anti-HIV (Rawal et al., 2005), anti-ischemic (Adachi et al., 1999), anticonvulsant (Ergenç et al., 1994), antimicrobial (Piscopo et al., 1989), antihistaminic (Previtera et al., 1994; Diurno et al., 1999), and antidiabetic agents (Carroll et al., 2011; Bruno et al., 2002), 15-hydroxy prostaglandin dehydrogenase inhibitors (Wu et al., 2011; Zidar et al., 2010), inhibitors of MurD ligase (Ha et al., 2012), aldose reductase inhibitors (Ma et al., 2012), for their anti-obesity effects (Bhattarai et al., 2010).

The thiazolidinediones affect genes and the specific gene expression profile induced by each of these molecules resulting in different activities for the thiazolidinedione derivatives. This observation has encouraged many medicinal and synthetic chemists to modify thiazolidinedione chemistry and utilize this core to synthesize numerous novel compounds with various pharmacological and therapeutic activities. The list of effects induced by the thiazolidinedione analogues is far from complete and needs specific attention. However, considering the broad therapeutic potential, this five-membered heterocyclic ring (thiazolidine-2,4-dione **4**) will play an important role in future

drug design and development.

The successful results by different researchers using thiazolidine core (Stachulski et al., 2011) encouraged us to identify new, potentially active anti-HBV agents. In this paper, we synthesized and evaluated the anti-HBV activity of different thiazolidone derivatives.

Materials and Methods

Chemistry: Chemicals and solvents were purchased either from Fluka or Merck, and all reagents were of analytical grade. Thin-layer chromatography (TLC) was performed on Merck silica gel 60 F₂₅₄ plates and visualized under UV light. ¹H NMR spectra were recorded with a Varian Mercury Plus 400 MHz instrument. ¹³C NMR spectra were recorded on a Varian Gemini 100 MHz instrument. Signals due to the solvent (¹³C NMR) or residual protonated solvent (¹H NMR) served as the internal standard. All the chemical shifts are reported in δ (ppm) using TMS as an internal standard. Multiplicity is indicated by one or more of the following abbreviations: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), br (broad); the coupling constants (J) correspond to the order of the multiplicity assignment. Mass spectra were recorded with a PE Sciex model API 3000 instrument. All the reactions were carried out under a nitrogen atmosphere.

Synthesis of 5-(ethoxymethylene)thiazolidine-2,4-dione (1): Pale yellow solid; yield: 98%; m.p.: 83–91°C; IR (KBr): ν_{max} 3383, 3137, 3043, 2989, 2432, 1721, 1677, 1473, 1392, 1229, 1105, 1010, 867, 740, 691 cm⁻¹; ¹H NMR (400 MHz, DMSO-d₆): δ 8.38 (br.s, 1H), 7.65 (s, 1H), 4.20 (q, J = 5.4 Hz, 2H), 1.40 (t, J = 5.4 Hz, 3H) ppm; ESI-MS: m/z 173.0 (M+1); ESI-HRMS: m/z calcd for C₆H₇NO₃S ([M+H]⁺) 174.0212, found 174.0219.

General experimental procedure for the synthesis of TZD derivatives (4a–4o): To a suspension of 1 (100 mg, 0.58 mmol) in acetonitrile was added secondary amines (0.58 mmol) in one lot. The reaction mixture was stirred at room temperature for 15–20 min, and the completion of the reaction was monitored by TLC. The precipitated solids were filtered at the pump and dried to obtain the pure compounds in quantitative yields.

(Z)-5-((Piperidin-1-yl)methylene)thiazolidine-2,4-dione (4a): White solid; yield: 98%; m.p.: 96–98°C; ¹H NMR (400 MHz, DMSO-d₆): δ 11.47 (br.s, 1H), 7.55 (s, 1H), 3.46–3.38 (m, 4H), 1.58–1.52 (m, 6H) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ 168.0, 167.5, 142.9, 85.1, 51.3(2C), 25.8 (2C), 23.2 ppm; ESI-HRMS: m/z calcd for C₉H₁₂N₂O₂S ([M+H]⁺) 213.0696, found 199.0692.

1-((Z)-(2,4-Dioxothiazolidin-5-ylidene)methyl)piperidine-4-carbonitrile (4b): White solid; yield: 98%; m.p.: 125–126°C; ¹H NMR (400 MHz, DMSO-d₆): δ 11.60 (br.s, 1H), 7.60 (s, 1H), 3.60–3.58 (m, 1H), 3.55–3.50 (m, 4H), 2.00–1.88 (m, 4H) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ 167.9,

167.6, 142.9, 121.5, 85.4, 40.3, 28.0(2C), 24.7 (2C) ppm; ESI-HRMS: m/z calcd for C₁₀H₁₁N₃O₂S([M+H]⁺) 238.0648, found 238.0645.

(Z)-5-((4-Ethylpiperazin-1-yl)methylene)thiazolidine-2,4-dione (4c): White solid; yield: 98%; m.p.: 88–89°C; ¹H NMR (400 MHz, DMSO-d₆): δ 11.50 (br.s, 1H), 7.56 (s, 1H), 3.45 (t, J = 5.6 Hz, 4H), 2.40 (t, J = 5.4 Hz, 4H), 2.32 (q, J = 4.8 Hz, 2H), 1.00 (t, J = 4.8 Hz, 3H) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ 167.9, 167.6, 142.7, 86.0, 52.0 (2C), 51.2 (2C), 49.7, 11.74 ppm; ESI-MS: m/z 242.1 (M+1); ESI-HRMS: m/z calcd for C₁₀H₁₅N₃O₂S ([M+H]⁺) 242.0962, found 242.0958.

tert-Butyl 4-((Z)-(2,4-dioxothiazolidin-5-ylidene)methyl)piperazine-1-carboxylate (4d): White solid; yield: 98%; m.p.: 72–78°C; ¹H NMR (400 MHz, DMSO-d₆): δ 11.56 (br.s, 1H), 7.60 (s, 1H), 3.46–3.38 (m, 8H), 1.40 (s, 9H) ppm; ¹³C NMR (400 MHz, DMSO-d₆): δ 167.9, 167.2, 153.5, 142.8, 86.7, 79.3 (2C), 49.3 (2C), 27.11 (4C) ppm; ESI-HRMS: m/z calcd for C₁₃H₁₉N₃O₄S ([M+H]⁺) 314.1170, found 314.1169.

(Z)-5-(((3,4-Dimethoxypyridin-2-yl)methyl)(methylamino)methylene)thiazolidine-2,4-dione (4e): White solid; yield: 98%; m.p.: 61–62°C; ¹H NMR (400 MHz, DMSO-d₆): δ 11.40 (br.s, 1H), 8.20 (s, 1H), 7.80 (s, 1H), 4.60 (s, 2H), 3.98 (s, 3H), 3.80 (s, 3H), 3.05 (s, 3H) ppm; ¹³C NMR (100 MHz, DMSO-d₆): δ 168.71, 167.54, 148.65, 145.7 (2C), 145.5, 142.8, 128.7, 108.4, 86.9, 60.4, 56.0 ppm; ESI-HRMS: m/z calcd for C₁₃H₁₅N₃O₄S ([M+H]⁺) 310.0852, found 310.0856.

(Z)-5-((Methyl(naphthalen-1-ylmethyl)amino)methylene)thiazolidine-2,4-dione (4f): Off-white solid; yield: 90%; m.p.: 81–82°C; ¹H NMR (400 MHz, CDCl₃): δ 11.58 (s, 1H), 8.02–7.84 (m, 4H), 7.62–7.58 (m, 3H), 7.40 (s, 1H), 5.10 (s, 2H), 3.15 (s, 3H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.5, 167.3, 144.6, 133.4, 131.4 (2C), 130.5, 128.7, 128.3 (2C), 126.6 (2C), 126.1 (2C), 125.5, 122.9, 87.0 ppm; ESI-HRMS: m/z calcd for C₁₆H₁₄N₂O₂S ([M+H]⁺) 299.0858, found 299.0849.

(Z)-5-((Methyl(3-phenyl-3-(4-(trifluoromethyl)phenoxy)propyl)amino)methylene)thiazolidine-2,4-dione (4g): White solid; yield: 88%; m.p.: 101–102°C; ¹H NMR (400 MHz, CDCl₃): δ 11.50 (s, 1H), 7.60–7.58 (m, 3H), 7.40–7.22 (m, 5H), 7.10 (s, 2H), 5.50 (s, 1H), 3.56–3.44 (m, 2H), 3.10 (s, 3H), 2.30–2.10 (m, 2H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.5, 167.2, 160.1, 144.4, 140.2, 128.6 (3C), 127.8, 126.7 (2C), 125.9(3C), 116.0 (3C), 76.7, 54.8, 36.2 ppm; ESI-HRMS: m/z calcd for C₂₁H₁₉N₂O₃F₃S ([M+H]⁺) 437.1160, found 437.1141.

(Z)-5-((Methyl(3-phenyl-3-(3-(trifluoromethyl)phenoxy)propyl)amino)methylene)thiazolidine-2,4-dione (4h): Pale yellow solid; yield: 90%; m.p.: 131–132°C; ¹H NMR (400 MHz, CDCl₃): δ 11.60 (s, 1H), 7.58–7.20 (m, 10H), 5.58 (s, 1H), 3.58–3.28 (m, 2H), 3.00 (s, 3H), 2.30–2.04 (m, 2H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 170.0, 157.5, 143.2,

140.3, 130.5, 130.2, 129.9 (2C), 128.6 (2C), 127.8 (2C), 126.0 (2C), 119.6, 117.2, 112.4, 109.5, 76.9 ppm; ESI-HRMS: m/z calcd for C₂₁H₁₉N₂O₃F₃S ([M+H]⁺) 437.1162, found 437.1141.

(Z)-5-((Methyl(3-phenyl-3-(o-tolylloxy)propyl)amino)methylene)thiazolidine-2,4-dione (**4i**): White solid; yield: 96%; m.p.: 129–130°C; ¹H NMR (400 MHz, CDCl₃): δ 11.60 (br.s, 1H), 7.58(s, 1H), 7.40–7.22 (m, 5H), 7.18–7.15 (m, 1H), 7.04–6.90 (m, 1H), 6.78–6.66 (m, 2H), 5.40(br.m, 1H), 4.22 (t, J = 6.6 Hz, 2H), 3.06 (s, 3H), 2.36 (s, 3H), 1.24 (t, J = 6.6 Hz, 2H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.6, 167.2, 155.1, 144.4, 141.2, 129.6 (2C), 129.4 (2C), 128.5 (3C), 127.6, 126.0 (2C), 115.7 (2C), 76.4, 36.6 (2C), 20.0 ppm; ESI-HRMS: m/z calcd for C₂₁H₂₂N₂O₃S ([M+H]⁺) 383.1403, found 383.1424.

(Z)-5-((Methyl(3-phenylpropyl)amino)methylene)thiazolidine-2,4-dione (**4j**): Pale yellow solid; yield: 90%; m.p.: 117–118°C; ¹H NMR (400 MHz, CDCl₃): δ 11.56 (br.s, 1H), 7.60 (s, 1H), 7.38–7.18 (m, 5H), 3.40 (t, J = 5.2 Hz, 2H), 3.10 (s, 3H), 2.62–2.50 (t, J = 7.6 Hz, 2H), 1.90–1.80 (m, 2H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.5, 167.2, 144.4, 141.0, 128.3 (3C), 128.2 (3C), 125.8, 31.8, 29.5 ppm; ESI-HRMS: m/z calcd for C₁₄H₁₆N₂O₂S ([M+H]⁺) 277.1014, found 277.1005.

(Z)-5-((4-((2-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)methylene)thiazolidine-2,4-dione (**4k**): White solid; yield: 89%; m.p.: 92–94°C; ¹H NMR (400 MHz, CDCl₃): δ 7.80 (d, J = 8 Hz, 1H), 7.52 (s, 1H), 7.40–7.20 (m, 8H), 4.80 (s, 1H), 3.50 (br.m, 4H), 2.46–2.20 (m, 4H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.7, 168.6, 142.1, 140.1, 139.2, 132.7, 129.6, 128.7, 128.6, 128.5, 128.1, 127.7, 127.4, 87.5, 69.5, 51.2, 49.6, 45.6, 40.1, 38.8, 11.40 ppm; ESI-HRMS: m/z calcd for C₂₁H₂₀N₃O₂SCl ([M+H]⁺) 414.1057, found 414.1038.

(Z)-5-((4-((4-Chlorophenyl)(phenyl)methyl)piperazin-1-yl)methylene)thiazolidine-2,4-dione (**4l**): Off-white solid; yield: 96%; m.p.: 108–109°C; ¹H NMR (400 MHz, CDCl₃): δ 11.60 (s, 1H), 7.58 (s, 1H), 7.56–7.20 (m, 5H), 4.40 (s, 1H), 3.42 (m, 4H), 2.38 (m, 4H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.1, 167.6, 142.6, 141.6, 141.2, 131.5 (2C), 129.4 (2C), 128.7 (2C), 128.6 (2C), 127.6, 127.2, 86.2, 73.2, 51.1 ppm; ESI-HRMS: m/z calcd for C₂₁H₂₀N₃O₂SCl ([M+H]⁺) 414.1008, found 414.1038.

(Z)-5-((4-(2,3-Dichlorophenyl)piperazin-1-yl)methylene)thiazolidine-2,4-dione (**4m**): White solid; yield: 96%; m.p.: 88–89°C; ¹H NMR (400 MHz, CDCl₃): δ 11.60 (s, 1H), 7.62 (s, 1H), 7.38 (m, 2H), 7.20 (dd, J = 6.6 Hz, 1H), 3.62–3.60 (m, 4H), 3.16–3.02 (m, 4H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.0, 150.3, 142.9, 132.6, 128.5 (2C), 126.1, 124.9, 120.0, 86.6, 50.7 (4C) ppm; ESI-HRMS: m/z calcd for C₁₄H₁₃N₃O₂SCl₂ ([M+H]⁺) 358.0186, found 358.0178.

(Z)-5-((4-(Benzo[d]isothiazol-3-yl)piperazin-1-yl)methylene)thiazolidine-2,4-dione (**4n**): Yellow solid; yield: 94%; m.p.: 118–119°C; ¹H NMR (400 MHz, CDCl₃): δ 11.30

(br.s, 1H), 8.10 (d, J = 8.4, 1H), 8. (d, J = 8.4, 1H), 7.70 (s, 1H), 7.58 (t, J = 7.2 Hz, 1H), 7.42 (t, J = 7.2 Hz, 1H), 3.76 (m, 4H), 3.58 (m, 4H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.1, 167.8, 162.7, 152.0, 142.9, 128.0, 127.0, 124.4 (2C), 124.1, 121.0, 86.8, 49.3 (4C) ppm; ESI-HRMS: m/z calcd for C₁₅H₁₄N₄O₂S₂ ([M+H]⁺) 347.0645, found 347.0631.

(Z)-5-((4-(5-Fluorobenzo[d]isoxazol-3-yl)piperidin-1-yl)methylene)thiazolidine-2,4-dione (**4o**): White solid; yield: 92%; m.p.: 115–117°C; ¹H NMR (400 MHz, CDCl₃): δ 11.58 (s, 1H), 8.15–8.07 (dd, J = 8.8, 1H), 7.76 (dd, J = 8.8 Hz, 1H), 7.60 (s, 1H), 7.30 (t, J = 6.8 Hz, 1H), 3.94–3.90 (m, 2H), 3.58–3.20 (m, 3H), 2.20–2.15 (m, 2H), 1.90–1.80 (m, 2H) ppm; ¹³C NMR (100 MHz, CDCl₃): δ 168.0, 164.9, 163.1, 162.4, 160.4, 143.0, 123.8, 117.0, 112.7, 97.4, 86.0, 32.4 (2C), 29.9 (3C) ppm; ESI-HRMS: m/z calcd for C₁₆H₁₄N₃O₃FS ([M+H]⁺) 348.0811, found 348.0813.

Biological evaluation

Cell culture: HepG2 2.2.15 cells, derived from HepG2 human hepatocellular carcinoma cells, were stably transfected with a head-to-tail HBV DNA dimer and were maintained in MEM with heat-inactivated 10% fetal bovine serum (FBS) and 1% antibiotics. In parallel experiments, human Huh7 hepatoma cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with heat-inactivated 10% FBS and 1% antibiotics. HepG2 2.2.15 and Huh7 cells were both grown at 37°C in a humidified atmosphere of 5% CO₂ and 95% air.

Cell viability assay: The cytotoxic effects of IN-4 (5) were determined using a Cell Titer 96 cell proliferation assay kit (MTS) (Promega, USA). In order to pinpoint the toxicity limits in HepG2 2.2.15 and Huh7 cells, they were plated into 96-well plates at a density of 4 × 10⁴ cells/mL for 24 hours. Cells were then treated with serial dilutions of IN-4 (5), ranging 2.5–160 mg/mL, and the mixture was incubated for 3 days. Cell toxicity was measured according to the manufacturer's protocol. All measurements were performed in four replicates, and results are presented as relative percentages over that of the control group.

Determination of HBV expression levels: After treating HepG2 2.2.15 cells or HBV-transfected Huh7 cells, levels of the HBsAg and HBeAg proteins were measured in culture media using an EIA kit (Johnson and Johnson, USA) according to the manufacturer's instructions.

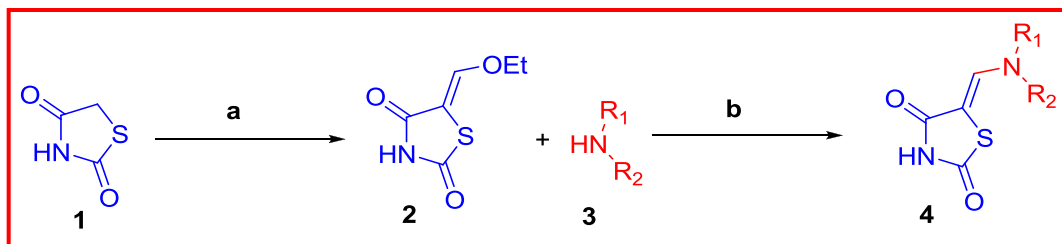
SEC general procedure: Capsid assembly was initiated by mixing Cp149 with test compounds in 2 buffer incubation 24 hours, respectively. Assembly reactions were examined on a Superose column (Biosep-SEC-S3000) mounted on HPLC system equipped with an auto injection module. The column was equilibrated with 100 mM HEPES pH 7.5, 300 mM NaCl at 0.6 mL/min.

Results and Discussion

The synthesis of a novel series of substituted 5-(aminomethylene)thiazolidine-2,4-diones (compounds **4a-4o**) is shown in Scheme 1. In the first step, thiazolidine-2,4-dione **1** was reacted with triethyl orthoformate in the presence of Ac_2O at reflux temperature afforded 5-(ethoxymethylene)thiazolidine-2,4-dione **2** as a pale yellow crystalline solid (Lo et al., 1954). The

condensation of **1** with various secondary amines in ethanol at reflux temperature resulted 5-(aminomethylene)thiazolidine-2,4-diones in good yields. The secondary amines were obtained from different commercial sources. Thus, the structures of the synthesized target compounds are listed in Table I.

Inhibition activities for HBV replication of the compounds **4a-4o** were determined in the HepG2.2.15



Scheme 1: Synthesis of 5-(aminomethylene)thiazolidine-2,4-diones (a) Ac_2O , $\text{HC}(\text{OEt})_3$, reflux (b) acetonitrile, 45°C , 30 min

Table I

Synthesis of a novel series of substituted 5-(aminomethylene)thiazolidine-2,4-diones^a

Entry	2° Amine (3)	Product	Yield ^b (%)
1			98
2			97
3			91
4			98

Table I

Continue

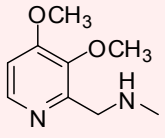
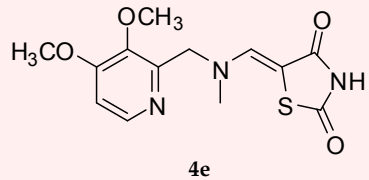
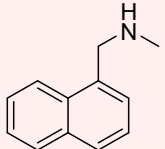
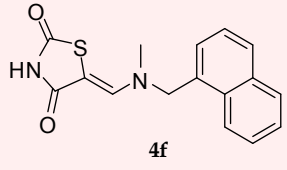
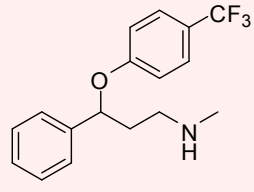
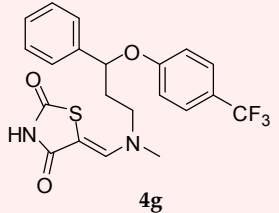
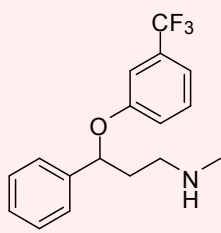
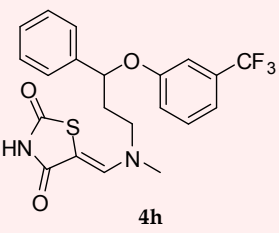
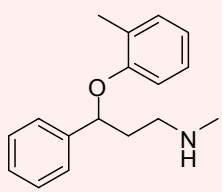
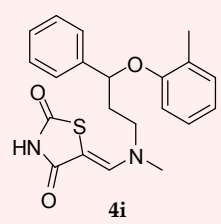
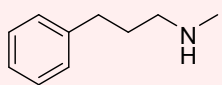
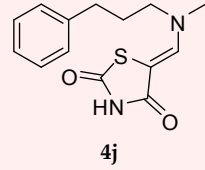
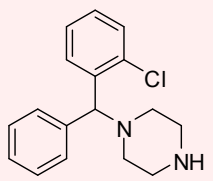
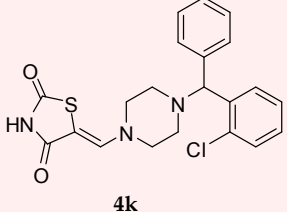
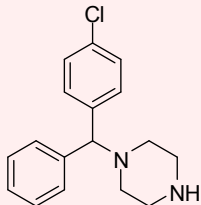
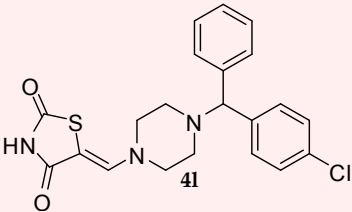
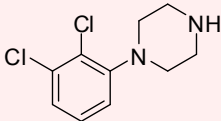
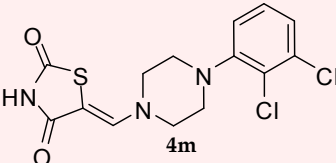
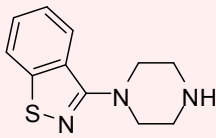
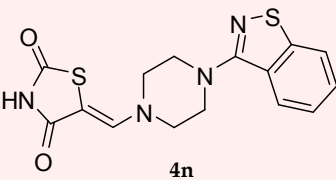
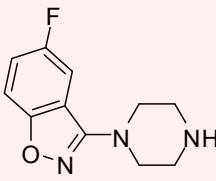
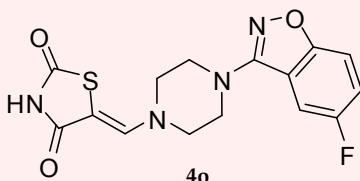
Entry	2° Amine (3)	Product	Yield ^b (%)
5		 4e	96
6		 4f	91
7		 4g	87
8		 4h	92
9		 4i	95
10		 4j	91
11		 4k	88

Table I

Continue

Entry	2° Amine (3)	Product	Yield ^b (%)
12			97
13			95
14			93
15			90

^aCompound 2 (1 mmol.), compound 3 (1 mmol.), acetonitrile (10 vol.), RT, 15-20 min; ^bIsolated yields

cells, which constitutively produces HBV genomes, and secretes virus-like particles (Korba et al., 1992). Lamivudine (3TC) was used as positive control. To ascertain the cytotoxic effects of the tested compounds, the cell viability was determined after the cells exposed to the compounds for 48 hours (Table II).

Hep G2.2.15 cell contained multiple copies of the HBV genome, which were stably integrated into the host cell genome and was widely used as a useful 'in vitro' model for evaluation of novel anti-HBV drugs. So, in the experiment, the Hep G2.2.15 cell line as in vitro cellular model was chosen.

All the target compounds were tested *in vitro* in HepG2.2.15 cells for anti-HBV activity and cytotoxicity. The properties of synthesized compounds are summarized in Table II, in which they are compared to the drug lamivudine. Compounds **4g**, **4h**, **4l**, **4m**, **4n**, and **4o** displayed good to better anti HBV activity.

A comparison of the IC₅₀ values of the derivatives **4a-4o**

clearly indicated that the size and polarity of substituent on the substitution had noticeable effect on its antiviral activities (Table II). **4l** (IC₅₀ = 6.66 μM), bearing a (Z)-5-((4-(4-chlorophenyl)(phenyl) methyl) piperazine group, was more potent inhibitor of HBV replication than 3TC (13.87 μM). When the attachments on TZD was replaced by rigidified piperazinyl derivatives to give compounds **4k**, **4m**, **4n**, **4o** (the IC₅₀ values 18.3, 6.3, 4.3 and 3.2 μM respectively), their anti-HBV activities were reserved or even improved. Replacing the piperazine attachment atom of N-methyl side chain containing nucleus in the compounds **4e** to **4j** there is no improvement in anti-HBV activity. There is no effect on the anti HBV activity when the TZD compound was substituted with piperidine or piperazine derivatives **4a** to **4d**. In the five 'piperazinyl' derivatives, **4k** (IC₅₀ = 18.3 μM), **4l** (IC₅₀ = 6.7 μM), **4m** (IC₅₀ = 6.3 μM), **4n** (IC₅₀ = 4.3 μM) and **4o** (IC₅₀ = 3.2 μM) also showed high inhibition of HBV replication, however, When there is no attachment on piperazine, such as **4c** and **4d**, both of them gave none antiviral

Table II				
Anti-HBV activity and cytotoxicity of target compounds <i>in vitro</i>				
Compound	Cytotoxic activity		Anti HBV activity	
	TC ₅₀ (μM)	TC ₀	IC ₅₀ (μM)	SI
4a	28.3	4.3	35.7	5.7
4b	32.7	4.9	27.6	3.2
4c	43.3	13.0	-	-
4d	77.2	10.3	-	-
4e	25.7	4.2	30.2	2.5
4f	46.8	12.8	9.6	-
4g	29.6	3.7	7.5	13.1
4h	96.4	14.7	6.7	6.2
4i	41.3	6.6	-	-
4j	31.2	4.6	14.3	12.3
4k	55.2	12.8	18.3	6.3
4l	54.0	14.0	6.7	8.1
4m	29.7	6.3	6.3	11.8
4n	25.8	5.9	4.3	12.5
4o	17.4	4.1	3.2	15.3
3TC	5.7	0.8	13.9 ± 1.4	

activity. It illuminated that the anti-HBV activity largely depends on the size, length and character of TZD substituent.

Among all the compounds **4m**, **4n**, and **4o** which are showing better anti-HBV activities, compounds containing the electron-withdrawing groups such as fluorine or chlorine are more effective than heterocyclic systems.

To gain better understand into the mechanisms of our compounds, **4o** was investigated to examine the effect on preformed HBV capsid and on HBV capsid assembly by size exclusion chromatography (Stray et al., 2005). Recovered protein was assigned to the void (aberrant capsid induced by **4o**, 8.5 min), capsid (9.5 min) and dimer (12.5 min) based on the HPLC chromatogram.

By SEC, we had observed the effect of compounds **4o** on Cp149 (Figure 1). There was no change of the capsid morphology detected at low concentration of **4o** (Cp149:4o = 4:1 or 2:1). At higher concentration (Cp149:4o = 1:2 or 1:4), the increasing continuous spectrum of void and the decreasing dimmers were observed. The results suggested that **4o** can break the equilibrium and change the product of HBV core protein self-assembly. By structural biology, we established a screening system for anti-HBV compounds that target on nucleocapsid. SEC would be a much better method to discover the strong antiviral compounds because of its objectivity, convenience and precision.

In conclusion, the newly synthesized TZD analogues **4a-4o** were evaluated for their anti-HBV activity, which provided 5 active derivatives inhibiting HBsAg secre-

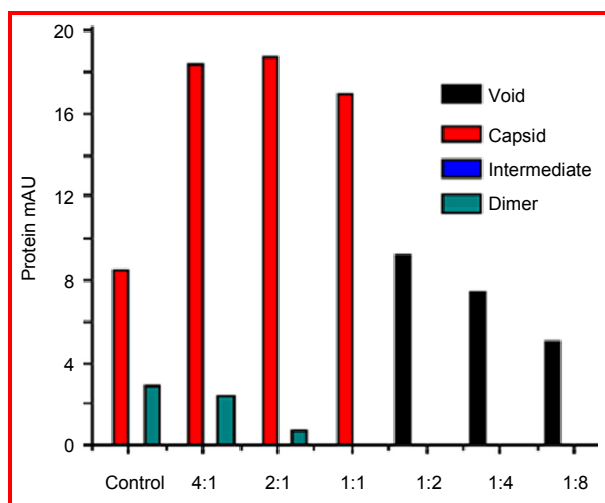


Figure 1: SEC showed the effect of Mo13 on capsid assembly

tion, 6 active derivatives suppressing HBeAg secretion and 5 active derivatives inhibiting HBV DNA replication. Interestingly, compound **4o** could inhibit not only HBsAg and HBeAg secretions but also HBV DNA replication with SI values of 41, 123.4 and 286. In view of their observed anti HBV activity, these compounds were seemed to have potential medicinal value.

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