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ARTICLE *in* EUROPEAN JOURNAL OF MEDICINAL CHEMISTRY · NOVEMBER 2014

Impact Factor: 3.45 · DOI: 10.1016/j.ejmech.2014.11.050 · Source: PubMed

European Journal of Medicinal Chemistry 90 (2015) 428–435

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Short communication

Design, synthesis, and bioevaluation of paeonol derivatives as potential anti-HBV agents

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ARTICLE INFO

Article history:
Received 3 September 2014
Received in revised form
22 November 2014
Accepted 25 November 2014
Available online 26 November 2014

Keywords:
Paeonol
HBV
Hepatitis B virus
Anti-HBV agent
Phenylsulfonyl group

ABSTRACT

Hepatitis B virus (HBV) is a causative reagent that frequently causes progressive liver diseases, leading to the development of acute, chronic hepatitis, cirrhosis, and eventually hepatocellular carcinoma (HCC). Despite several antiviral drugs including interferon-α and nucleotide derivatives are approved for clinical treatment for HBV, critical issues remain unresolved, e.g., low-to-moderate efficacy, adverse side effects, and resistant strains. In this study, novel Paeonol-phenylsulfonyl derivatives were synthesized and their antiviral effect against HBV was evaluated. The experimental results indicated that these compounds process significant antiviral potential, including the inhibition of viral antigen expression and secretion, and the suppression of HBV viral DNA replication. Among compounds synthesized in this research, compound 2-acetyl-5-methoxyphenyl 4-methoxybenzenesulfonate (**7f**) had the most potent inhibitory activity with IC_{50} value of 0.36 μM, and high selectivity index, SI (TC_{50}/IC_{50}) 47.75; which exhibited an apparent inhibition effect on viral gene expression and viral propagation in cell culture model. So, we believe our compounds could serve as reservoir for antiviral drug development.

1. Introduction

Hepatitis B virus (HBV), a member of the family *Hepadnaviridae*, is a causative agent, frequently leading to acute and chronic infections in humans [1]. HBV infections are a major global health problem, despite the availability of an effective vaccine. Worldwide, over two billion people are infected with HBV at present, and approximately 400 million are chronically infected carriers [2,3]. Furthermore, 80% of chronic HBV carriers have varying degrees of liver damage, which could progress to liver cirrhosis and hepatocellular carcinoma [4]. Although antiviral reagents such as interferons and nucleotide analogs have been widely used to treat chronically infected patients, the rapid development of resistance and the undesirable side effects of the reagents indicate that it is imperative to urgently develop an antiviral drug to treat HBV infection [5–8].

After infection by HBV, four viral transcripts are transcribed from four different viral promoters (Core, X, S, and PreS); the transcripts have lengths of 3.5, 2.4, 2.1, and 0.8 kb, respectively. The 3.5-kb transcript is translated to produce core, precore, and viral polymerase proteins. It also serves as a pregenomic RNA template for viral genome synthesis. The 2.4- and 2.1-kb transcripts encode small, medium, and large surface (envelope) proteins; the 0.8-kb

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http://dx.doi.org/10.1016/j.ejmech.2014.11.050
0223-5234/

transcript is translated to produce the X protein, a potent transactivator for both viral and host gene promoters [9]. In addition, instead of focusing on viral genome replication, recent studies have determined how the viral gene is regulated and controlled, with the objective of ameliorating the disease; this is a crucial step toward devising antiviral strategies for curing hepatitis B [10,11]. The discovery of novel and effective antiviral agents is usually the result of (1) understanding the viral lifecycle [12] and (2) performing molecular modeling by using the coordinates of the crystal structures of viral proteins such as helicases, proteases, and polymerases [13]. Such targeted drug development has been lacking for HBV, and the most common strategy has been compound screening by using in vitro replication models [10,11,14,15]. Once a new compound that inhibits HBV has been identified, the next step is to determine its mode of antiviral action.

At present, several antiviral compounds have been reported to target cellular signaling factors for interfering with viral gene regulation and genome replication [10,11]. In molecular virology, the regulation of HBV viral gene expression is a highly complex topic, and understanding how viral proteins regulate the host signaling network can help control the virus propagation.

It had been reported that some anti-HBV agents were nucleoside, nucleotide, or derivatives from botanical origin [16–18]. Paeonol, 2-hydroxy-4-methoxy acetophenone (**1**, Fig. 1), has been a major constituent of Traditional Chinese medicine for more than a thousand years, and it is obtained from peony. Paeonol is a flavonoid derivative that exhibits several interesting biological activities, and it has been used as an anti-inflammatory [19,20], analgesic [20], antioxidant [21], antidiabetic [22], and acaricidal agent [23]. Paeonol is also a promising candidate for neuroinflammatory therapy [24] or a radiosensitizer for lung adenocarcinoma [25]. Recently, Lee reported the antiatherogenic action of paeonol resulting from the upregulation of the nuclear translocation of liver X receptor α (LXRα), which enhances the mRNA and protein expression of ATP-binding membrane cassette transport protein A1 (ABCA1) and reduces cholesterol accumulation [26]. Hsieh reported that the paeonol can protect the memory after an ischemic stroke by reducing Amyloid precursor protein (APP), beta-site APP cleaving enzyme (BACE), and apoptosis [27]. Moreover, paeonol derivatives are associated with several biological activities. Yang presented paeonol Schiff-base derivatives (**2**, **3**, Fig. 1) that could form complexes with copper ions and that showed high antioxidant activity, moderate DNA-binding activity, and excellent tumor cell cytotoxicity [28]. Yu reported that paeonol thiosemicarbazone derivatives (**4**, **5**, Fig. 1) are potential mushroom tyrosinase inhibitors [29]. In this paper, we present a new series of paeonol derivatives with a phenylsulfonyl side chain. The paeonol core structure was retained and derivatized with a new phenylsulfonyl side chain on the phenol group. The paeonol derivatives were evaluated for antiviral activity, and they were observed to show potential anti-HBV effects in HepG2 2.2.15 cells. The synthesized compounds are potential structural templates for designing and developing novel anti-HBV agents.

2. Results and discussion

2.1. Synthesis

It has been reported that introducing a phenylsulfonyl moiety in the molecules could increase the solubility of the molecules and trigger antitumor activity [30–32]. To obtain various paeonol-phenylsulfonyl derivatives, we treat paeonol with substituted phenylsulfonyl chloride to get the desired compounds, which are shown in Scheme 1. All these products were obtained in good yields and purified to more than 95% by recrystallization for anticancer assays.

2.2. Cytotoxic effect of the compounds on HepG2 2.2.15 hepatoma cells

To evaluate the structure–activity relationship (SAR) and determine the cytotoxic effects of compounds **7a**–**h** on HepG2 2.2.15 cells, the cells were subjected to twofold serial dilution with each compound for 72 h, and the viability of the cells was measured according to the manufacturer's protocol. All measurements were performed in four replicates, and the results were presented as a relative percentage of the results of the control group. The results are shown in Table 1; a decrease in the cell viability in a dose-dependent manner was observed for all compounds. In Table 1, the TC_{50} values of compounds **7c**, **7d**, **7e**, **7f**, **7g**, and **7h** show a potent cytotoxic effect. Compounds **7a** and **7b** did not show cytotoxic activity, and F and H, which were "bioisosteres" having similar physical properties or biological effects, were substituted; however, the substituted F typically reduced the overall lipophilicity of the molecules [33]. Furthermore, Cl and Br were another set of bioisosteres corresponding to compounds **7c** and **7d;** these compounds showed almost identical activity. However, they showed higher cytotoxicity than **7a** and **7b**. Compound **7g** showed higher cytotoxicity (10.92 μM) than **7h** (68.76 μM); both these compounds were nitrogen-substituted compounds. Compound **7g** was a

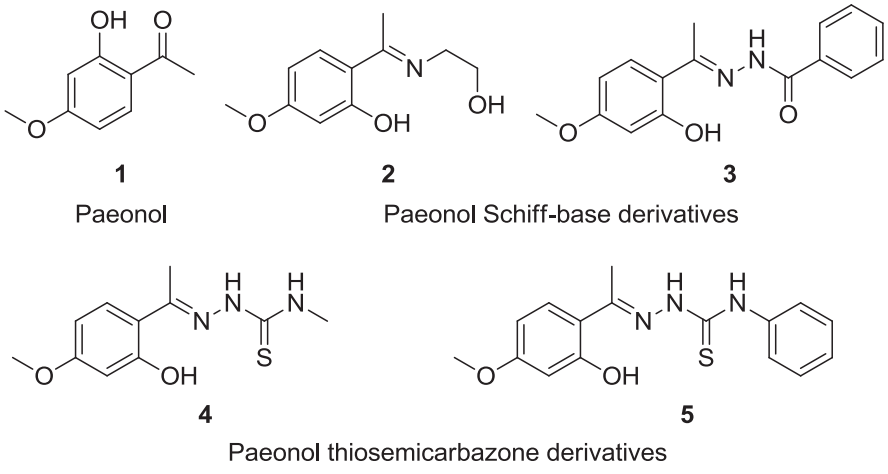
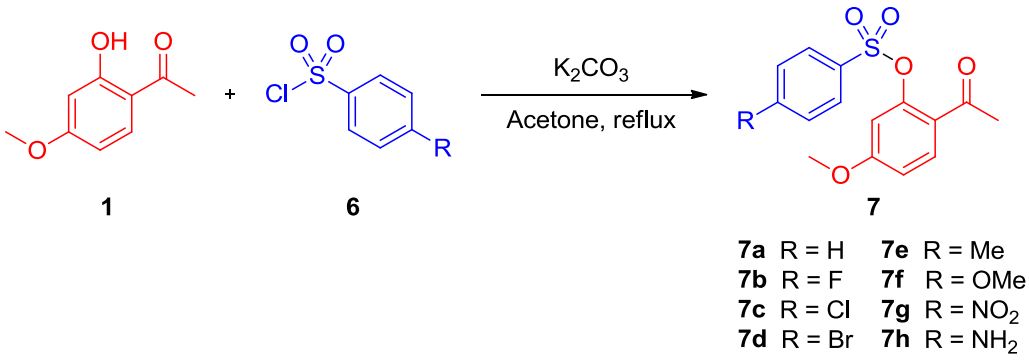


Fig. 1. Structures of Paeonol, Paeonol Schiff-base derivatives, and Paeonol thiosemicarbazone derivatives.



Scheme 1. Synthesis of Paeonol-phenylsulfonyl derivatives.

lipophilic NO_2 substitute, whereas the other was a hydrophilic NH_2 group. The optimal non-cytotoxic drug concentrations were then used in antiviral assays.

2.3. Antiviral effect of compounds on HBV viral antigen expression in HepG2 2.2.15 cell culture medium

Fig. 2 shows the secretion of HBsAg and HBeAg in treated HepG2 2.2.15 cell culture media. Production of HBsAg and HBeAg was dramatically decreased by compounds **7a**–**g**. Only the maximal concentration of **7h** can decrease both HBsAg and HBeAg expression and secretion (Fig. 2). In addition, compound **7f** has a potent inhibitory effect on both HBsAg and HBeAg viral antigen expression and secretion.

To further confirm the effect of 7**f** on HepG2 2.2.15 growth, we assessed their morphology of growth response by Giemsa staining method. The general cellular morphology after 7**f** treatment is shown in Fig. 3A. The morphology of HepG2 2.2.15 cells did not have significant different between treated compound 7**f** and control group (Fig. 3A). To further test the role of compound 7**f** in HepG2 2.2.15 cell cycle progression, we performed flow cytometry analysis. DNA of the HepG2 2.2.15 cells were stained by PI to analysis population of DNA content as index of cell cycle after treated with 7**f** (Fig. 3B). Dose-dependent of 7**f** did not decrease ratio of cells in each phase of cell cycle. According to these results suggest that 7**f** did not affect HepG2 2.2.15 cell in growth and cell cycle.

2.4. Antiviral effect of compounds on HBV DNA replication in HepG2 2.2.15 cells

To investigate the antiviral activity of compounds in HBV DNA production, HepG2 2.2.15 cells were treated with three non-cytotoxic concentrations of compounds for 48 h. The cultural media were harvested and the viral genomic DNA of the secreted virion particle was isolated using real-time PCR analysis. As shown in Fig. 4, the results revealed that during treatment, compounds **7a**–**g** could attenuate various levels of viral DNA in the secreted virion particle. However, compound **7h** has no effect on the viral DNA production. In addition, compound **7f** exhibits a significant inhibitory effect on the secreted viral DNA level, and the IC_{50} or selectivity index value of this compound on viral DNA replication is better than the commercial antiviral drug lamivudine (3TC) (Tables 1 and 2). However, lamivudine has no apparent effect on the viral HBsAg and HBeAg expression (Table 1).

2.5. Effect of compound 2 on HBV viral gene expression

For **7f**, which showed a potent inhibitory activity for HBV, we examined its antiviral effect on viral gene expression. HepG2 2.2.15 cells were treated with three non-cytotoxic concentrations of compound **7f** for 48 h, and the total cellular RNA was extracted and subjected to Northern blot analysis of HBV viral RNA levels. As shown in Fig. 5, treatment of **7f** significantly decreased the levels of 3.5-kb precore/pregenomic and 2.4-/2.1-kb surface antigen RNA in a dose-dependent manner.

3. Conclusion

Various phenylsulfonyl side chains were directly conjugated to the Traditional Chinese medicine paeonol through chemical synthesis to generate eight new series of paeonol-phenylsulfonyl derivatives, which were potential anti-HBV leads. The substituents on the phenylsulfonyl side chain included F, Cl, Br, NO_2, Me, OMe, and NH_2. All the synthesized compounds were characterized and evaluated for their anti-HBV activities. This is the first ever report dealing with the mechanism by which paeonol-phenylsulfonyl derivatives inhibit HBV gene expression and viral DNA replication. We found a novel molecular mechanism regulated by paeonol-phenylsulfonyl derivatives in hepatocyte. The results showed that **7f** exhibited potent activity against HBV in HepG2 2.2.15 cells with IC_{50} values of 0.36 μg/mL, especially for the high selectivity index (TC_{50}/IC_{50}) of 47.75, and the compound could therefore be used a lead compound in anti-HBV therapy.

4. Experimental

4.1. Chemistry

All reactions were carried out in oven-dried glassware (120 °C) under an atmosphere of nitrogen. Acetone, ethyl acetate and hexane from Mallinckrodt Chemical Co. were dried and distilled from CaH_2. Benzenesulfonyl chloride, 4-bromobenzenesulfonyl chloride, 4-chlorobenzenesulfonyl chloride, 4-fluorobenzenesulfonyl chloride, 4-methoxybenzenesulfonyl chloride, 4-nitrobenzenesulfonyl chloride, peaonol (2′-hydroxy-4-methoxyacetophenone), potassium carbonate and *p*-toluenesulfonyl chloride were purchased from Sigma–Aldrich Chemical Co without further purification.

Analytical thin layer chromatography (TLC) was performed on precoated plates (silica gel 60 F–254), purchased from Merck Inc. Purification by gravity column chromatography was carried out by use of Merck Reagents Silica Gel 60 (particle size 0.063–0.200 μmm, 70–230 mesh ASTM). Infrared (IR) spectra were measured on a Bruker ALPHA-P FTIR spectrometer. Absorption

Table 1
Effect of compounds **7a**–**h** on cytotoxicity of HepG2 2.2.15 cells, and on inhibition potential of HBV viral antigen and DNA replication for 72 h treatment.

Compound	TC_{50} (μM)	HBsAg IC_{50} (μM)	HBeAg IC_{50} (μM)	Inhibition of HBV DNA replication IC_{50} (μM)
7a	55.37	7.3	16.49	1.86
7b	53.72	5.05	11.18	2.71
7c	13.77	2.83	4.2	0.57
7d	10.08	2.01	1.86	0.44
7e	23.72	3.18	6.83	1.04
7f	17.19	1.55	2.24	0.36
7g	10.92	1.77	4.64	1.15
7h	68.76	21.67	72.45	–
5-FU[a]	21.68	–	–	–
Lamivudine (3TC)[a]	352.03	–	–	7.63

TC_{50}: the concentration of the compound at which cell viability was reduced to 50%. IC_{50}: the concentration of the compound on anti-HBV effect was reached to 50%.
[a] 5-FU: Fluorouracil, is the positive control for cytotoxic analysis Lamivudine (3TC) is the positive control for anti-HBV analysis.

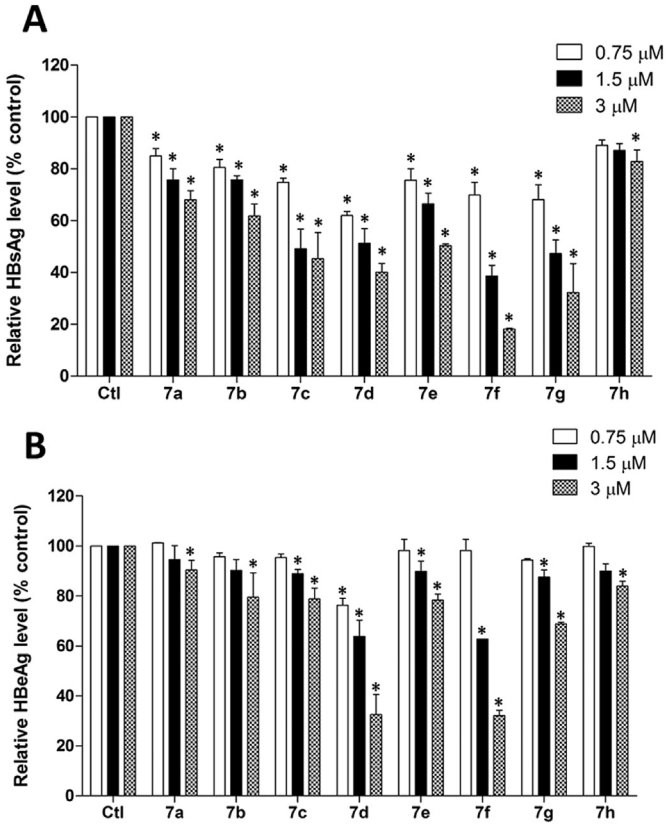


Fig. 2. Effects of compounds **7a**–**h** on HBV viral (A) HBsAg and (B) HBeAg secretion. HepG2 2.2.15 cells were treated with three concentrations (0.75, 1.5 and 3.0 μM) of compounds **7a**–**h** or treated with DMSO 0.1% (v/v) (control) for 72 h. The cultural media of each treatment were collected for viral HBsAg and HBeAg EIA analysis. The data are expressed as the mean and the standard deviation of the mean. ($n = 3$, $^*p < 0.05$ vs untreated cells).

intensities are recorded by the following abbreviations: s, strong; m, medium; w, weak. Proton NMR spectra were obtained on a Bruker Advance 500 (500 MHz) by use of chloroform-*d* as solvent. Proton NMR chemical shifts are referenced to the $CHCl_3$ singlet (7.24 ppm). Carbon-13 NMR spectra were obtained on a Bruker Avance 500 (125 MHz) by use of chloroform-*d* as solvent. Carbon-13 chemical shifts are referenced to the center of the $CDCl_3$ triplet (77.0 ppm). Multiplicities are recorded by the following abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; *J*, coupling constant (hertz). High-resolution mass spectra were obtained by means of a FINNIGAN/MAT-95XL mass spectrometer. High-performance liquid chromatography (HPLC) analyses were carried out by Agilent 1100 series system with CNW Athena C18 column (120 Å, 4.6 mm × 250 mm, 5 μm) and UV detection at 254 nm.

4.1.1. Standard procedure for the synthesis of aryl-sulfonate from peaonol with benzenesulfonyl chloride derivatives

To a reaction vessel containing peaonol (1.00 equiv.), benzenesulfonyl chloride derivatives (1.20 equiv.) and potassium carbonate (2.00 equiv.) in 20.0 mL acetone was stirred and refluxed for 12.0 h. It was quenched with water and removed acetone under reduced pressure. The residue was extracted with ethyl acetate (3 × 30 mL). The combined organic layer were washed with brine, dried over $MgSO_{4(S)}$, filtered, and concentrated under reduced pressure. The crude product was purified by column chromatography on silica gel (ethyl acetate and hexane as eluant) to give desired products. All the products with purity of >95.0% were checked by HPLC.

*4.1.2. 4′-Methoxy-2′-[(phenylsulfonyl)oxy]acetophenone **(7a)***

A orange viscous oil (83% yield); IR (ATR, cm^{-1}) 3069 (w), 3012(w), 2841 (w), 1683 (m), 1607 (s), 1376 (s), 1257 (s), 1195 (s), 1120 (s), 1063 (s), 952 (m), 794 (s), 686 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 7.83 (dd, $J_{AB} = 8.25$ Hz, $J_{CD} = 1$ Hz, 2H), 7.67–7.65 (m, 2H), 7.52 (t, $J = 7.5$ Hz,2H), 6.81 (dd, $J_{EF} = 9$ Hz, $J_{GH} = 2.5$ Hz, 1H), 6.57 (d, $J = 2.5$ Hz,1H), 3.73 (s, 3H), 2.43 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.1, 163.1, 148.9, 135.0, 134.6, 131.9, 129.3, 128.5, 125.7, 113.0, 108.5, 55.7, 30.3; HRMS (EI) calculated for $C_{15}H_{14}O_5S$, 306.0562, found 306,0556.

*4.1.3. 2′-[(4-Fluorophenylsulfonyl)oxy]-4′-methoxy-acetophenone **(7b)***

A white solid (82% yield); IR (ATR, cm^{-1}) 2962 (w), 2924 (w), 1680 (s), 1609 (w)m 1591 (m), 1491 (m), 1377 (s), 1258 (s), 1239 (s), 1183 (s), 1151 (m), 951 (s), 791 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 7.89–7.86 (m, 2H), 7.67 (d, $J = 9$ Hz, 2H), 7.21 (t, $J = 8$ Hz, 2H), 6.83 (dd, $J_{AB} = 8.5$ Hz, $J_{CD} = 2.5$ Hz, 1H), 6.65 (d, $J = 2.5$ Hz, 1H), 3.79 (s, 3H), 2.44 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.9, 163.1, 148.5, 132.0, 131.5, 131.4, 125.5, 116.7, 116.6, 112.8, 108.8, 55.7, 30.0; HRMS (EI) calculated for $C_{15}H_{13}FO_5S$, 324.0468, found 324.0458.

*4.1.4. 2′-[(4-Chlorophenylsulfonyl)oxy]-4′-methoxy-acetophenone **(7c)***

A white solid (75% yield); IR (ATR, cm^{-1}) 3019 (w), 2922 (w), 2897 (w), 1680 (s), 1608 (s), 1566 (m), 1373 (s), 1256 (s), 1238 (m), 1183 (s), 1090 (s), 952 (s), 871 (s), 622 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 7.77 (d, $J = 8.5$ Hz, 2H), 7.66 (d, $J = 9$ Hz, 1H), 7.49 (d, $J = 9$ Hz, 2H), 6.82 (dd, $J_{AB} = 9$ Hz, $J_{CD} = 2.5$ Hz, 1H), 6.61 (d, $J = 2.5$ Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.9, 163.1, 148.5, 141.4, 133.5, 132.1, 130.0, 129.6, 125.5, 112.9, 108.8, 55.8, 30.1; HRMS (EI) calculated for $C_{15}H_{13}ClO_5S$, 340.0172, found 340.0170.

*4.1.5. 2′-[(4-Bromophenylsulfonyl)oxy]-4′-methoxy-acetophenone **(7d)***

A white solid (75% yield); IR (ATR, cm^{-1}) 3093 (w), 2951 (w), 2850 (w), 1680 (s), 1609 (s), 1370 (s), 1256 (s),1183 (m), 1150 (s), 952 (s), 798 (s), 609 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 7.70–7.64 (m, 5H), 6.81 (dd, $J_{AB} = 8.5$ Hz, $J_{CD} = 2$ Hz, 1H), 6.59 (d, $J = 2$ Hz, 1H), 3.75 (s, 3H), 2.42 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.8, 163.1, 148.4, 134.0, 132.6, 132.0, 130.0, 129.9, 125.4, 112.8, 108.7, 55.7, 30.0; HRMS (EI) calculated for $C_{15}H_{13}BrO_5S$, 383.9967, found 383.9674.

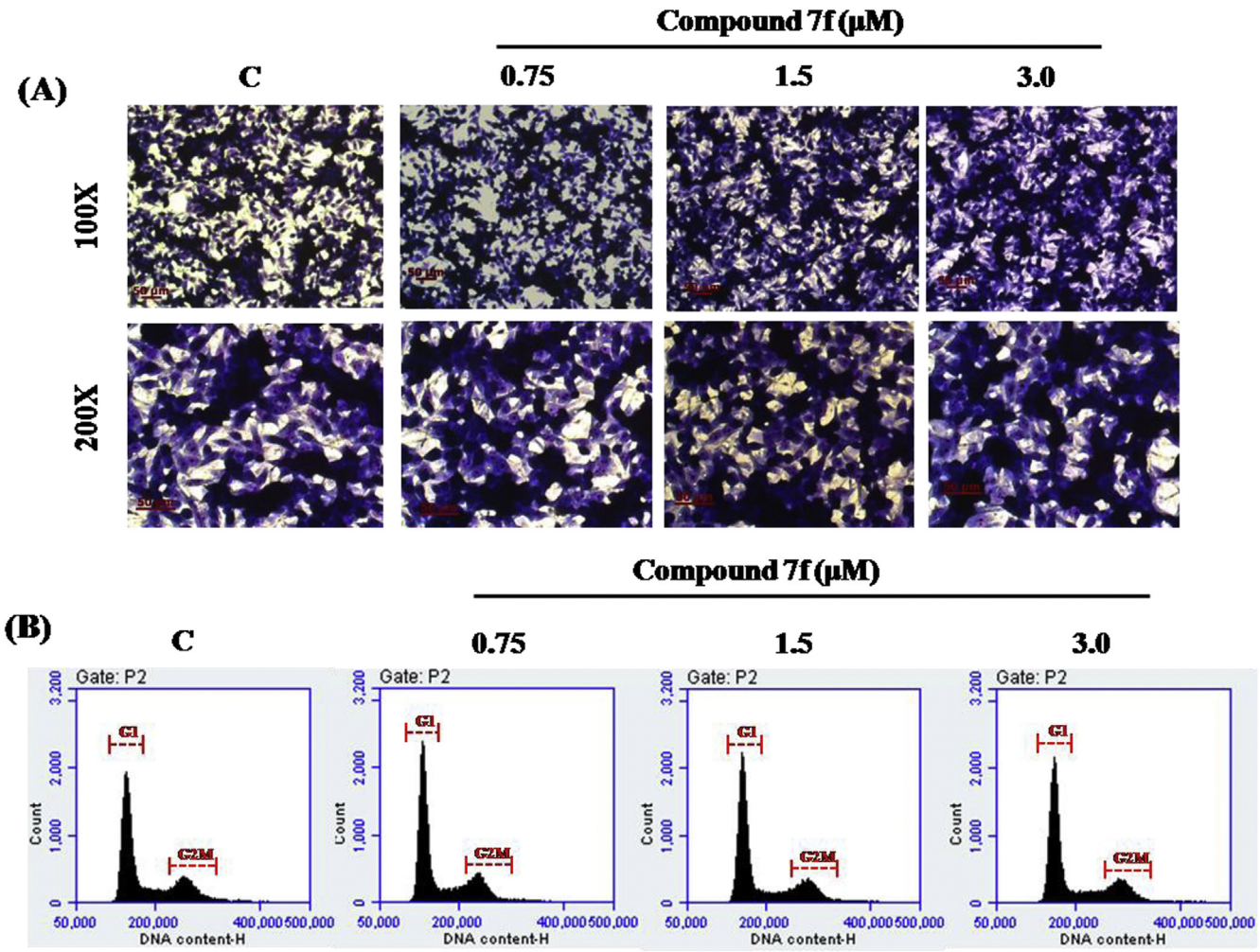


Fig. 3. Effect of **7f** on cellular morphology and DNA content analysis of HepG2 2.2.15 cells. HepG2 2.2.15 cells were exposed to three non-cytotoxic concentration (0.75, 1.5 and 3.0 μM) of **7f**. After 72 h of culture, cells were fixed and stained with Giemsa's solution to identify morphology of HepG2 2.2.15 cells. (A) By cellular morphology, cell inhibition of HepG2 2.215 cells growth was not observation on the cells treated **7f**. (B) Cell cycle distribution analysis of HepG2 2.2.15 in treated **7f** was performed by flow cytometry.

*4.1.6. 4′-Methoxy-2′-[(p-tolylsulfonyl)oxy]acetophenone **(7e)***

A yellow solid (77% yield); IR (ATR, cm^{-1}) 2922 (w), 2862 (w), 2844 (w), 1681 (s), 1609 (m), 1368 (s), 1322 (m), 1257 (s), 1238 (m), 1193 (s), 1152 (s), 971 (s), 784 (s), 717 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 7.67–7.62 (m, 3H), 7.28 (d, J = 8 Hz, 2H), 6.77 (dd, J_{AB} = 8.5 Hz, J_{CD} = 2.5 Hz, 1H), 6.55 (d, J = 2 Hz, 1H), 3.70 (s, 3H), 2.41 (s, 3H), 2.40 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.0, 163.0, 148.9, 145.9, 131.9, 131.8, 129.8, 128.4, 125.6, 112.7, 108.5, 55.6, 30.2, 21.6; HRMS (EI) calculated for $C_{16}H_{16}O_5S$, 320.0718, found 320.0718.

*4.1.7. 4′-Methoxy-2′-[(4-methoxyphenylsulfonyl)oxy]-acetophenone **(7f)***

A white solid (81% yield); IR (ATR, cm^{-1}) 2972 (w), 2848 (w), 1667 (m), 1595 (m), 1564 (m), 1496 (m), 1412 (s), 1268 (s), 1172 (s), 835 (s), 780 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 7.68 (d, J = 8.5 Hz, 2H), 7.61 (d, J = 9 Hz, 1H), 6.91 (d, J = 8.5 Hz, 2H), 6.75 (dd, J_{AB} = 8.5 Hz, J_{CD} = 1.5 Hz, 1H), 6.53 (t, J = 2.5 Hz, 1H), 3.81 (s, 3H), 3.69 (s, 3H), 2.41 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.0, 164.3, 163.0, 148.9, 131.8, 130.7, 125.9, 125.6, 114.4, 112.6, 108.5, 55.7, 55.6, 30.2; HRMS (EI) calculated for $C_{16}H_{16}O_6S$, 336.0668, found 336,0672.

*4.1.8. 4′-Methyloxy-2′-[(4-nitrophenylsulfonyl)oxy]acetophenone **(7g)***

A yellow solid (78% yield); IR (ATR, cm^{-1}) 3105 (w), 3072 (w), 2922 (w), 2853 (w), 1671 (s), 1600 (s), 1528 (s), 1364 (s), 1354 (s), 1313 (s), 1193 (s), 1120 (s), 1059 (s), 933 (m), 870 (m), 803 (s), 747 (m), 658 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 8.35 (dd, J_{AB} = 7 Hz, J_{CD} = 2 Hz, 2H), 8.08 (dd, J_{AB} = 7 Hz, J_{CD} = 2 Hz, 2H), 7.67 (d, J = 9 Hz, 1H), 6.85 (dd, J_{EF} = 8.5 Hz, J_{GH} = 2.5 Hz, 1H), 6.67 (d, J = 2.5 Hz, 1H), 3.79 (s, 3H), 2.41 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 195.6, 163.2, 151.0, 147.9, 140.7, 132.3, 130.0, 125.1, 124.3, 112.9, 109.2, 55.8, 29.7; HRMS (EI) calculated for $C_{15}H_{13}NO_7S$, 351.0413, found 351.0412.

*4.1.9. 2′-[(4-Aminophenylsulfonyl)oxy]-4′-methyloxy-acetophenone **(7h)***

7g (100.0 mg, 0.295 mmol) was dissolved in 10.0 mL ethanol. And the reaction mixture was added 10% Pd/C (7.6 mg). Then the reaction mixture was stirred at room temperature for 12.0 h. The mixture was filtered, and the filtrate was purified by column chromatography on silica gel (ethyl acetate and hexane as eluant) to give desired products. A yellow solid (86% yield); IR (ATR, cm^{-1}) 3473 (w), 3356 (w), 3239 (w), 2923 (s), 2853 (s), 1595 (s), 1464 (s), 1347 (s); ^1H NMR (500 MHz, $CDCl_3$): δ 7.66 (d, J = 9 Hz, 1H), 7.52 (d, J = 7 Hz, 2H), 6.78 (dd, J_{AB} = 9 Hz, J_{CD} = 3 Hz, 1H), 6.64–6.60 (m,

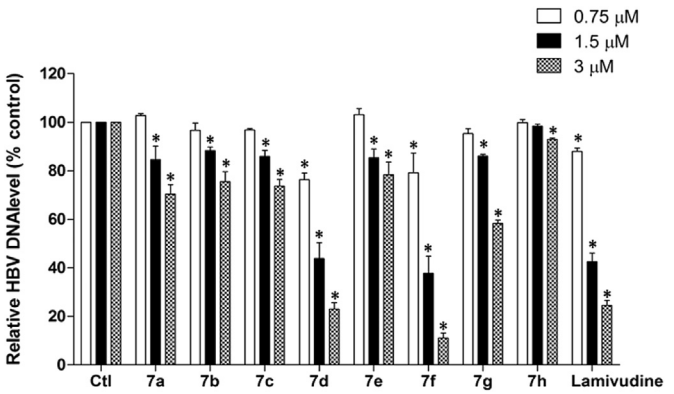


Fig. 4. Effects of compounds **7a**–**h** on HBV DNA replication in HepG2 2.2.15 cells. Cells were treated with three concentrations (0.75, 1.5 and 3.0 μM) of each compound for 72 h or treated with DMSO 0.1% for 72 h and the culture media of each treatment were collected for viral DNA extraction. The detection of viral DNA was carried out by Real-time PCR analysis. The data are expressed as the mean and the standard deviation of the mean. ($n = 3$) (*$p < 0.05$ vs untreated cells).

Table 2
The selectivity index (SI) of anti-HBV of compounds **7a**–**h**.

Compound	HBsAg SI (TC_{50}/IC_{50})	HBeAg SI(TC_{50}/IC_{50})	HBV DNA replication SI(TC_{50}/IC_{50})
7a	7.58	3.36	29.77
7b	10.64	4.81	19.82
7c	4.87	3.28	24.16
7d	4.99	5.42	22.91
7e	7.46	3.47	22.81
7f	11.09	7.67	47.75
7g	6.17	2.35	9.5
7h	3.17	0.95	–
Lamivudine (3TC)[a]	–	–	46.13

[a] Lamivudine (3TC) is the positive control for anti-HBV analysis.

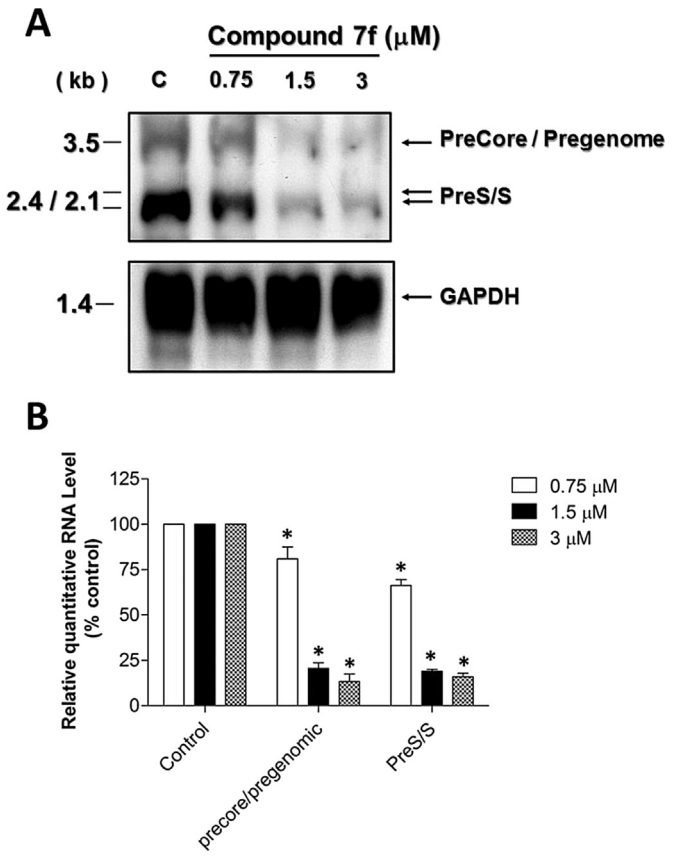


Fig. 5. Effects of **7f** on HBV RNA expression in HepG2 2.2.15 cells. (A) HepG2 2.2.15 cells were treated with three non-cytotoxic concentrations (0.75, 1.5 and 3.0 μM) of **7f** for 48 h and the total cellular RNA was extracted and subjected to Northern blot analysis. (B) The intensity of each RNA band was quantitated with densitometer and the relative amount was normalized with GAPDH loading control. The data shown are representative of three replicate experiments. (*$p < 0.05$ vs untreated cells).

3H), 4.34 (b, 2H), 3.76 (s, 3H), 2.46 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$): δ 196.7, 163.2, 152.3, 149.5, 131.8, 130.9, 125.9, 122.0, 113.8, 112.9, 108.6, 55.7, 29.7; HRMS (EI) calculated for $C_{15}H_{15}NO_5S$, 321.0671, found 321.0675.

4.2. Biology

4.2.1. Cell culture and reagents

HepG2 2.2.15 cells derived from HepG2 human hepatocellular carcinoma cells and stably transfected with a head-to-tail HBV DNA dimer [34] were maintained in MEM with heat-inactivated 10% fetal bovine serum (FBS) and 1% antibiotics and grown at 37 °C in a humidified atmosphere of 5% and 95% air. The synthesized compounds were dissolved in DMSO (Sigma–Aldrich). The final concentration of DMSO in all reactions was maintained at 0.1% in all experiments. Morphology of cell was assessed by microscopy and Giemsa staining method. For Giemsa staining, monolayer of cells was rinsed twice with PBS, fixed in methanol for 10 min and stained for 10 min with Giemsa stain solution (Gibco). Distain cells with water until back ground stain is removed. Morphology of cell was photographed by microscopy (Axio Vert. A1; ZIESS).

4.2.2. Cell viability assay

The cytotoxic effect of compounds was determined by a CellTiter 96® AQ_{ueous} one solution cell proliferation assay kit (MTS) (Promega) to pinpoint the non-toxic test compound concentration in HepG2 2.2.15 cells. Briefly, HepG2 2.2.15 cells were plated into 96-well plates at a density of 4×10^4 cells/ml for 24 h. The cells were then treated with serial dilutions of compounds for 3 days, and the toxicity of cells was measured according to the manufacturer's protocol. All measurements were performed in four replicates, and the results are presented as relative percentages over that of the control group.

4.2.3. Flow cytometry

Cell cycle analysis of compound 7**f** was determined by propidium iodide (PI) staining followed by flow cytometry in HepG2 2.2.15 cells. HepG2 2.215 cells were seeded into 6-cm dishes at a density of 2×10^5 cells/dish for 24 h. Cells were then treated with serial dilution of compound 7**f** for 72 h. The cells were harvested and fixed with ice cold 70% ethanol at 4 °C for 24 h. After the washes, cells were followed by staining with PI staining solution (20 μg/mL PI in 0.3 mL of PBS containing 200–400 unit of RNase A) followed by incubation at 37 °C for 30 min in the dark. The cells were anaylzed by flow cytometry (Accuri C6, Becton Dickenson) to determine the proportion of cells within cycle.

4.2.4. Determination of viral HBsAg and HBeAg antigens

After treating the HepG2 2.2.15 cells, the levels of the viral surface antigen (HBsAg)and e antigen (HBeAg) were measured in the culture media using an enzyme immunoassay (EIA) kit (Johnson and Johnson), according to the manufacturer's instructions.

4.2.5. Real-time PCR analysis of HBV DNA level during treatment

The quantity of viral DNA in the medium was determined by Real-time PCR analysis by using NucleoSpin Blood mini-kit (Macherey-Nagel) for viral DNA extraction. The forward primer was 5′- AGGAGGCTGTAGGCATAAATTGG -3′ and the reverse primer was 5′- CAGCTTGGAGGCTTGAACAGT-3′ [35] were synthesized for PCR to detected viral genome. The PCR reactions were performed using SYBR Green PCR master mix and the primer pair with the following program: initial denaturation at 50 °C for 2 min and 95 °C for 10 min, followed by 45 cycles of amplification at 95 °C for 15s and annealing/extending at 58 °C for 1 min [36].

4.2.6. Analysis of intracellular HBV-RNA by Northern blotting

Total RNA from treated cells was extracted using the Trizol® isolation buffer (Invitrogen) according to the manufacturer's protocol. Five micrograms of total RNA was denatured, separated on a 1.0% agarose gel using commercial kit (Amresco), and transferred to a positively charged Hybond-N+ nylon membrane (Amersham). After UV cross-linking, the membrane was hybridized with a DIG-labeled full-length HBV genome probe, washed, and exposed to X-ray film. Briefly, the full-length HBV probe was produced by restriction enzymatic digestion of the pHBV2 plasmid with *Eco*RI [37], purified, labeled with a DIG high prime DNA labeling kit (Roche, Mannheim). The total RNA amount was normalized by hybridization of the membrane with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe, which was produced by *Pst* I digestion of the pGAPDH plasmid (provided by Dr. Hsiao-Sheng Liu from the Graduate Institute of Microbiology and Immunology, National Cheng Kung University, Tainan, Taiwan).

4.2.7. Statistical analysis and quantification of data

The data were expressed as the mean and the standard deviation (SD) of the mean from three independent experiments using GraphPad Prism (GraphPad Software Inc.). Variance analysis and the Student's *t*-test were used for data analysis. Differences were considered significant when $P < 0.05$. Quantitative data from Northern blot analysis were obtained using the computing densitometer and TotalLab Quant software (Nonlinear Dynamics Ltd.).

Acknowledgments

For financial support, we thank the Ministry of Science and Technology (103-2113-M-007-020) of the Republic of China National Tsing Hua University, and China Medical University Hospital. This work was supported by Ministry of Science and Technology of Taiwan (Grant NSC-102-2113-M-007-010) and China Medical University Hospital Research Foundation, Taiwan (DMR-103-042) (DMR-103-071). The authors express heartfelt thanks to Dr. Pei-Jer Chen for HepG2 2.2.15 cells as model for antiviral research. We also thank the Laboratory Animal Core Facility which is funded by Agriculture Biotechnology Research Center (ABRC) at Academia Sinica for technical support in cell morphology and flow cytometry experiments.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2014.11.050.

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