Phytochemistry 99 (2014) 107–114

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Synthesis and antiviral effects of isosteviol-derived analogues against the hepatitis B virus

Tsurng-Juhn Huang [a], Bo-Hon Chou [a,b,e], Cheng-Wen Lin [c], Jen-Hsien Weng [a], Chang-Hung Chou [a], Li-Ming Yang [b,d], Shwu-Jiuan Lin [b,*]

[a] Department of Biological Science and Technology and Research Center for Biodiversity, China Medical University, Taichung 404, Taiwan
[b] Department of Pharmaceutical Sciences, School of Pharmacy, Taipei Medical University, Taipei 110, Taiwan
[c] Department of Medical Laboratory Science and Biotechnology, China Medical University, Taichung 404, Taiwan
[d] Division of Chinese Medicinal Chemistry, National Research Institute of Chinese Medicine, Ministry of Health and Welfare, Taipei 112, Taiwan
[e] Long Shine Biopharma Co., Tainan 722, Taiwan

ARTICLE INFO

Article history:
Received 10 May 2013
Received in revised form 21 June 2013
Available online 23 January 2014

Keywords:
Isosteviol derivative
Hepatitis B virus
Human hepatoma cells
NF-κB
TLR2

ABSTRACT

Among several isosteviol-derived analogues, NC-8 (*ent*-16-oxobeyeran-19-*N*-methylureido) showed inhibitory potency against the hepatitis B virus (HBV) in HepG2 2.2.15 cells. Its anti-HBV mechanism was then next investigated in a human hepatoma cell culture system. Results showed that it specifically inhibited viral gene expression and reduced the level of encapsidated viral DNA intermediates in Huh7 cells that expressed replicating HBV. It also potently attenuated all viral promoter activity in HBV-expressing Huh7 cells, but not in cells lacking HBV expression. By examining its antiviral mechanism in cellular signaling pathways, NC-8 was found to inhibit the activity of the nuclear factor (NF)-κB element-containing promoter, but only slightly enhanced activities of activator protein (AP)-1- and interferon-sensitive response element (ISRE)-containing promoters in HBV-expressing cells. NC-8 also significantly eliminated NF-κB (p65/p50) and Toll-like receptor (TLR)2 proteins, but increased the IκBα protein level in a dose-dependent manner in HBV-transfected Huh7 cells, while these protein levels were apparently unchanged in non-transfected cells. Meanwhile, NC-8-treated nuclear extracts that co-expressed HBV inhibited the binding of NF-κB to the CS1 site of HBV major surface gene and specifically attenuated CS1-containing promoter activity. Taken together, this study suggests that the antiviral mechanism of NC-8 appears to be mediated by disturbing replication and gene expression of HBV and by inhibiting the host TLR2/NF-κB signaling pathway.

Introduction

Hepatitis B virus (HBV), a member of the Hepadnaviridae, is a causative agent that frequently leads to acute and chronic infections in humans (Gitlin, 1997). Approximately 80% of HBV chronic carriers have varying degrees of liver destruction which can promote liver cirrhosis and hepatocellular carcinoma (HCC) (Park et al., 2006). As non-human hosts of HBV are restricted to only a few species of animals, such as the chimpanzee, Peking duck, woodchuck, and ground squirrel, the development of efficient therapeutics for HBV infection has been hampered (Chisari et al., 1987). Despite the development of a recombinant vaccine against HBV and the use of specific HB immunoglobulin in susceptible populations, over 400 million people are chronic carriers (Gish, 2005). After infection, four viral transcripts are transcribed from four different viral promoters (Core, X, S, and PreS), which are 3.5, 2.4, 2.1, and 0.8 kb long, 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 the small, middle, and large surface (envelope) proteins; the 0.8-kb transcript is translated to produce the X protein, a potent transactivator in both viral and host gene promoters (Chisari, 2000). Determining how to regulate viral gene expression, instead of viral replication, is a crucial issue for antiviral strategies. Several antiviral drugs including interferon (INF)-α and nucleoside derivatives are approved for clinical treatment of HBV (Pramoolsinsup, 2002), yet critical issues remain unresolved, e.g., low-to-moderate efficacy, adverse side-effects, and resistant strains (Wakui et al., 2010). In light of these facts, continued development of new antiviral agents with novel targets and mechanisms to eradicate HBV in chronic carriers is urgently needed.

Stevioside is a natural sweetener that exists in leaves of *Stevia rebaudiana* (Bertoni) Bertoni (Compositae) (Geuns, 2003). Isosteviol, an *ent*-beyerane diterpenoid that possesses many biological activities (Chang et al., 2008), can readily be obtained by acid

* Corresponding author. Tel.: +886 2 27361661x6133; fax: +886 2 27390671.
E-mail address: shwu-lin@tmu.edu.tw (S.-J. Lin).

0031-9422/$ - see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.phytochem.2013.12.014

hydrolysis of stevioside (Avent et al., 1990). Because natural products can provide discovery of molecular targets for developing drugs, a number of isosteviol analogues obtained from biocatalysis and/or chemical modification showed bioactive attributes, including anti-inflammation (Chang et al., 2009), inhibition of α-glucosidase, a cytotoxic effect, and antibacterial activity (Zhu et al., 2013). Recently, Takasaki et al. (2009) reported that isosteviol displayed a significant inhibitory effect against early antigen activation of the Epstein–Barr virus. A literature survey indicated that replacement of the carboxylic group on triterpenoids by a ureide moiety had antiviral activity towards the influenza and HSV-1 viruses (Baltina et al., 2003; Flekhter et al., 2003). Thus, to develop new anti-HBV agents with novel targets and mechanisms, several isosteviol derivatives were prepared by replacing the 19-COOH with the ureide moiety, and their inhibitory action against HBV in HepG2 2.2.15 cells were evaluated. Among them, NC-8 (**2**) (*ent*-16-oxobeyeran-19-*N*-methylureido, Scheme 1) was found to be effective against HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) secretion. Its IC_{50} of inhibition to HBsAg secretion was 7.89 μg/ml, which was more potent than the positive control lamivudine (49.13 μg/ml). Encouraged by the results, the anti-HBV molecular mechanism of NC-8 (**2**) was examined using a human hepatoma cell culture system. Herein, described are the synthesis and results of anti-HBV testing, as well as the mode of antiviral action of NC-8 (**2**).

Results

Synthesis and screening for anti-HBV activity

The preparation of compounds **2**–**17** was carried out as outlined in Scheme 1. Initially, the intermediate of isocyanate **1**, which was previously synthesized (Militsina et al., 2007), was treated with the appropriate amines in dimethylformamide (DMF) in the presence of triethylamine at room temperature to afford **2**–**17** (Scheme 1). These were structurally identified and elucidated based on NMR and HRESIMS analyses. Subsequently, the synthesized compounds were evaluated against hepatitis B virus in HepG2 2.2.15 cells. NC-8 (**2**) was effective against HBsAg and HBeAg secretion (Table 1), prompting further investigation of its effect using a human hepatoma cell culture system.

Cytotoxicity of NC-8 to Huh7 cells

Fig. 1 shows that NC-8 (**2**) caused significant cytotoxic effects only at doses of >40 μg/ml in HBV-transfected Huh7 cells. However, it did not exhibit significant cytotoxicity in non-transfected cells (data not shown) at these concentrations. In the following experiments, a non-cytotoxic dose (<40 μg/ml) was used for antiviral treatment of transfected cells.

Table 1
Anti-HBV activity of isosteviol derivatives in vitro[a]

Compound	TC_{50}[b]	HBsAg[c]		HBeAg[d]	
		IC_{50}[e] (μg/ml)	SI[f]	IC_{50} (μg/ml)	SI[f]
2 (NC-8)	53.52	7.89	6.78	24.30	2.20
10	70.30	–	–	–	–
14	125.86	–	–	24.83	5.06
16	75.18	26.13	2.88	9.36	8.03
5-FU[g]	6.12	–	–	–	–
Lamivudine[h]	205	49.13	4.17	–	–

[a] Compounds **3–9**, **11–13**, **15**, and **17** were not active against HBV in HepG2 2.2.15 cells.
[b] TC_{50}: 50% cytotoxic concentration in HepG2 2.2.15 cells.
[c] HBsAg: HBV surface antigen.
[d] HBeAg: HBV e antigen.
[e] IC_{50}: 50% inhibitory concentration.
[f] SI (selectivity index) = TC_{50}/IC_{50}.
[g] 5-FU (fluorouracil): positive control of cytotoxicity.
[h] Lamivudine as the positive control.

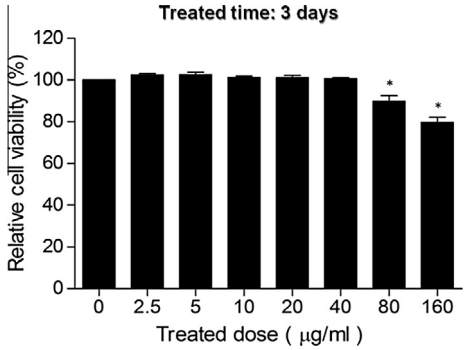
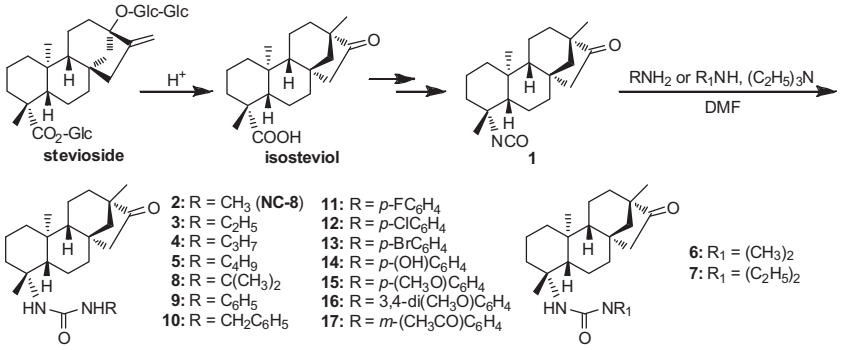


Fig. 1. Effect of NC-8 on Huh7 cytotoxicity. Huh7 cells were transfected with the pHBV1.2 plasmid for 48 h, and then treated with NC-8 (**2**) (at doses of 2.5–160.0 μg/ml) for 3 days. Its cytotoxicity was determined by an MTS assay. ($n = 4$) (*$p < 0.05$ vs. untreated cells).

Inhibition of HBV HBsAg and HBeAg secretion and viral DNA intermediates by NC-8

Fig. 2A shows secretion of HBsAg and HBeAg in culture media of treated Huh7 cells. Production of HBsAg and HBeAg dramatically dropped with NC-8 (**2**) treatment in a dose-dependent manner compared to the vehicle control ($p < 0.05$). The EC_{50} of NC-8 (**2**) on HBsAg and HBeAg secretion were 4.8 and 5.9 μg/ml, respectively. To investigate its antiviral activity on HBV DNA production, Huh7 cells were transiently transfected with the pHBV1.2 viral genome and treated with NC-8 (**2**) for 48 h. Cells were harvested,



Scheme 1. The Synthetic route to compounds **2–17**.

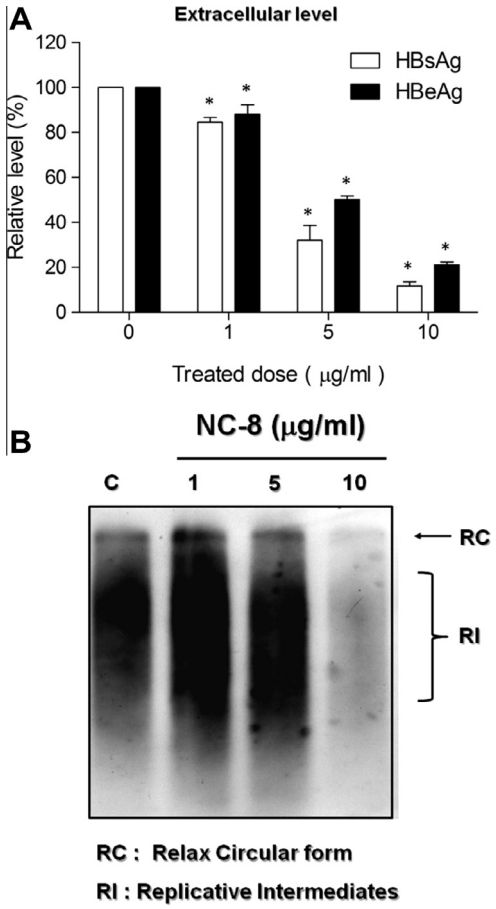


Fig. 2. Effects of NC-8 on production of HBV surface antigen (HBsAg), HBV e antigen (HBeAg), and HBV DNA levels of viral core particles in Huh7 cells. (A) Huh7 cells were seeded on 6-well plates (3×10^5 cells/well) in DMEM with 10% FBS and incubated overnight. Cells were transfected with the pHBV1.2 plasmid for 2 days, and then treated with NC-8 (**2**) at non-cytotoxic concentrations (0, 1, 5, and 10 μg/ml) in medium containing 2% FBS for another 2 days. Culture media were then collected for HBsAg and HBeAg viral antigen EIA analyses. Data are expressed as the standard error of the mean. ($n = 3$) (*$p < 0.05$ vs. untreated cells). (B) Huh7 cells plated on 60-mm culture dishes (6×10^5 cells/dish) were transfected and treated with various concentrations of NC-8 (**2**) in 2% FBS-containing DMEM for 2 days. Cells were harvested, and total cellular genomic DNA was extracted and analyzed by a Southern blot analysis using HBV DNA as the probe as described in "Experimental". Data shown are representative of three sets of experiments.

and total genomic DNA was isolated. Fig. 2B shows that NC-8 (**2**) attenuated the production of HBV viral relaxed circular DNA and intermediates at doses of >5 μg/ml.

Antiviral activity of NC-8 in HBV-transfected Huh7 cells and viral promoter activities

The antiviral activity of NC-8 (**2**) was examined in the HBV-transfected Huh7 cell line that stably replicated HBV (the adw2 serotype). HBV RNA and viral protein levels were determined by Northern and Western blot analyses after NC-8 (**2**) treatment. As shown in Fig. 3A, expression levels of the 3.5-kb precore/pregenomic RNA were slightly reduced during treatment, but the 2.4/2.1-kb surface antigen RNA was strongly inhibited by NC-8 (**2**) in a dose-dependent manner. Consistently, treatment of NC-8 (**2**) specifically decreased protein levels of three forms of the surface antigen (LHBs, MHBs, and SHBs) and HBV core protein (HBcAg) in a dose-dependent manner (Fig. 3B). Furthermore, four viral promoter-reporter plasmids were cloned and constructed for promoter activity assays to understand its molecular mechanism. These constructs corresponding to the viral core protein (pCore-

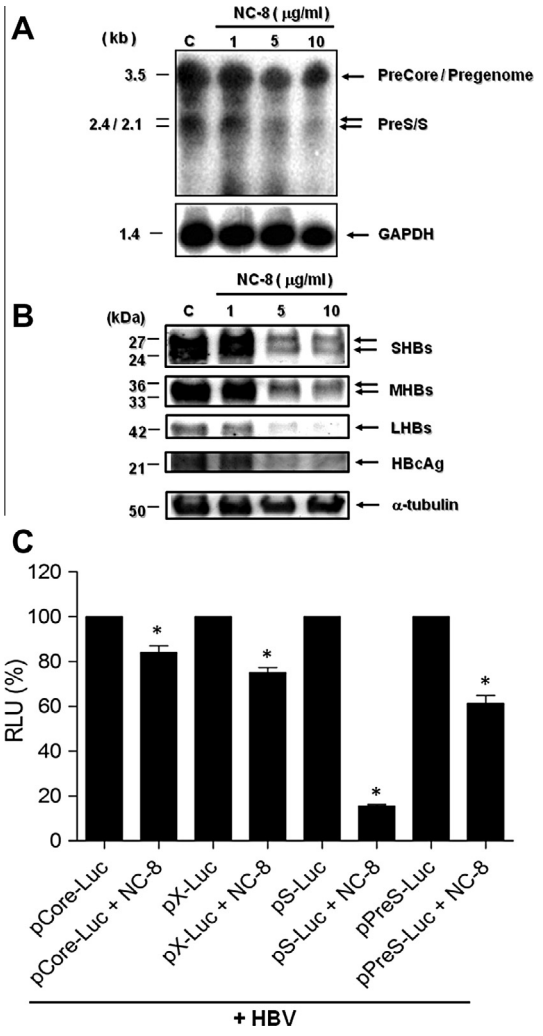


Fig. 3. Inhibitory effects of NC-8 on the expression of hepatitis B virus (HBV) genes and viral promoter activity in Huh7 cells. Huh7 cells were plated on 60-mm dishes. Transfection and treatment with NC-8 (**2**) were conducted as described in Fig. 2B. Treated cells were harvested and subjected to (A) total RNA and (B) protein isolation. (A) Total RNA from transfected and treated Huh7 cells was subjected to a Northern blot analysis using HBV DNA as the probe as described in "Experimental". GAPDH served as an RNA loading control. (B) Total proteins from whole-cell lysates were separated by SDS–PAGE, followed by a Western blot analysis to detect three HB surface proteins (LHBs, MHBs, and SHBs) and viral core protein (HBcAg). α-Tubulin served as the protein loading control. Data shown are representative of three sets of experiments. (C) Huh7 cells were transfected with either a pCore-Luc, pX-Luc, pS-Luc, or pPreS-Luc HBV gene promoter-luciferase reporter construct together with a pHBV1.2 HBV whole-genome expression plasmid and pRL-SV40 for 24 h. After transfection, cultures were treated with or without NC-8 (**2**) (10 μg/ml) for 2 days. Cells were lysed and subjected to a dual-luciferase assay. Data are expressed as relative levels of luciferase activity compared to the NC-8 untreated control. ($n = 3$) (*$p < 0.05$ vs. untreated cells).

Luc), major viral surface antigen (pS-Luc), viral large surface antigen (pPreS-Luc), and viral X protein (pX-Luc) were co-transfected with the pHBV1.2 plasmid in Huh7 cells. Treatment with NC-8 (**2**) at a dose of 10 μg/ml strongly suppressed activities of the surface antigen gene promoter (S and PreS) and slightly decreased those of the core and X promoter ($p < 0.05$) (Fig. 3C). However, treatment of HBV non-expressing cells with NC-8 (**2**) had no effect on any viral promoter activities (data not shown).

Effects of NC-8 on host cellular signaling pathways

To determine how NC-8 (**2**) inhibits the effect of HBV on host cellular signaling pathways, three luciferase reporters containing

binding elements of either NF-κB, AP-1, or the ISRE were co-transfected with the pHBV1.2 plasmid or a control vector into Huh7 cells. After treatment with NC-8 (**2**) at a dose of 10 μg/ml, cells were harvested and subjected to a promoter activity assay. Fig. 4A shows that NC-8 (**2**) specifically inhibited NF-κB-containing promoter activity but enhanced AP-1- and ISRE-containing (Fig. 4B, C, respectively) promoter activity ($p < 0.05$) when the HBV was expressed in cells. To our surprise, treatment of HBV-negative Huh7 cells with NC-8 (**2**) had the same effect on promoter activities. The latter activities of NC-8 (**2**)-treated HBV non-expressing cells were lower in the NF-κB reporter, but higher in the AP-1 and ISRE reporters compared to untreated control.

Effects of NC-8 on NF-κB, IκB, and TLR2 protein expressions in HBV-transfected or non-transfected Huh7 cells

In view of the key role of the NF-κB signaling pathway in HBV gene regulation and viral genome replication in HBV infection (Doria et al., 1995; Hildt et al., 1996; Kwon and Rho, 2002; Lin et al., 2009; Meyer et al., 1992), Huh7 cells-transfected with or without the pHBV1.2 plasmid were treated with NC-8 (**2**) followed by Western blot analysis. Fig. 5A shows that HBV non-transfected Huh7 cells expressed a low level of NF-κB, and NC-8 (**2**) treatment (10 μg/ml) did not affect its expression. In contrast, expressions of the two forms of the NF-κB protein (p65/p50) significantly increased in Huh7 cells transfected with the HBV genome. Nonetheless, increasing the treated dose of NC-8 (**2**) of HBV-expressing Huh7 cells greatly reduced NF-κB protein levels (Fig. 5A). To compare correspondence with the expression of NF-κB, the NF-κB inhibitor of the IκBα protein was also detected by an immunoblot analysis. Results showed that treatment of HBV-negative Huh7 cells with NC-8 (**2**) slightly decreased IκBα protein expression. However, transfection of HBV into cells dramatically reduced IκBα protein levels. In addition, treatment of HBV-expressing cells with NC-8 (**2**) significantly increased expression of IκBα in a dose-dependent manner (Fig. 5A). By contrast, TLR2 was previously detected in Huh7 cells (Preiss et al., 2008), which was speculated to be an activator of NF-κB in the cellular innate immune response during HBV infection (Thompson et al., 2009). Thus, the expression of TLR2 was examined by immunoblotting to determine the potential factors of the NF-κB signaling pathway regulated by NC-8 (**2**). Fig. 5B shows that expression levels of TLR2 protein were not altered by NC-8 (**2**) in HBV non-transfected Huh7 cells. In cells transfected with the HBV genome, expression of the TLR2 protein significantly increased, whereas NC-8 (**2**) treatment dramatically reduced its levels in a dose-dependent manner.

Effect of NC-8 on DNA-binding activity of NF-κB in the HBV surface gene

Several putative NF-κB-binding sites were identified in the HBV genome (Lin et al., 2009). Among them, a specific NF-κB-binding site had a positive regulatory role in HBV gene expression. The site is located in the HBV genome at nt207 to 216 (CS1) which corresponds to the viral surface gene coding region. To examine whether NC-8 (**2**) regulated viral gene expression by NF-κB as mediated through the CS1 site, nuclear extracts were prepared from cells with and without HBV transfection and were tested for the presence of NF-κB to CS1 site-binding activity by an EMSA. The results showed that transfection of the HBV genome significantly increased the binding activity of NF-κB to the HBV CS1 site. However, NC-8 (**2**) slightly decreased NF-κB DNA-binding activity in non-HBV-transfected nuclear extracts. In contrast, NC-8 (**2**) dramatically reduced the DNA-binding activity of NF-κB to the CS1 site (Fig. 6A). To further examine the possibility of an effect of NC-8 (**2**) on HBV CS1-containing promoter activity, p5CS1-Luc was constructed and transfected into Huh7 cells with or without HBV expression. The transfections were then treated with or

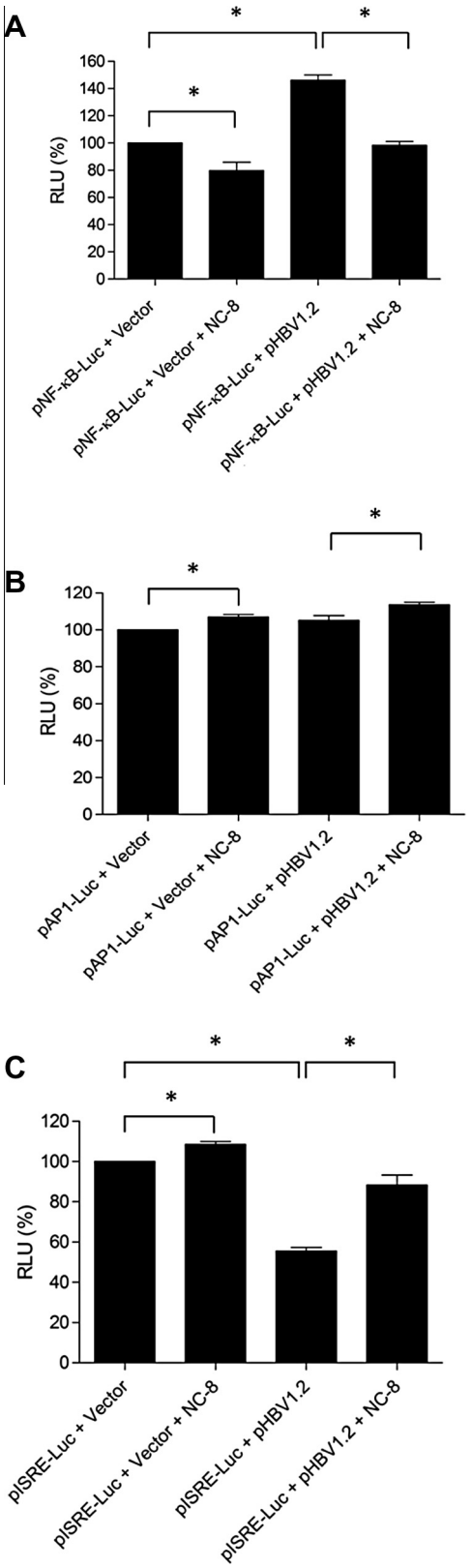


Fig. 4. Cellular signaling pathways were modulated by NC-8. Huh7 cells were transfected with either (A) pNF-κB-Luc, (B) pAP1-Luc, or (C) pISRE-Luc promoter–reporter constructs together with or without the pHBV1.2 plasmid and treated with or without NC-8 (**2**) as described in Fig. 3. Data are expressed as relative levels of luciferase activity compared to the NC-8 (**2**) untreated control. ($n = 3$) (*$p < 0.05$).

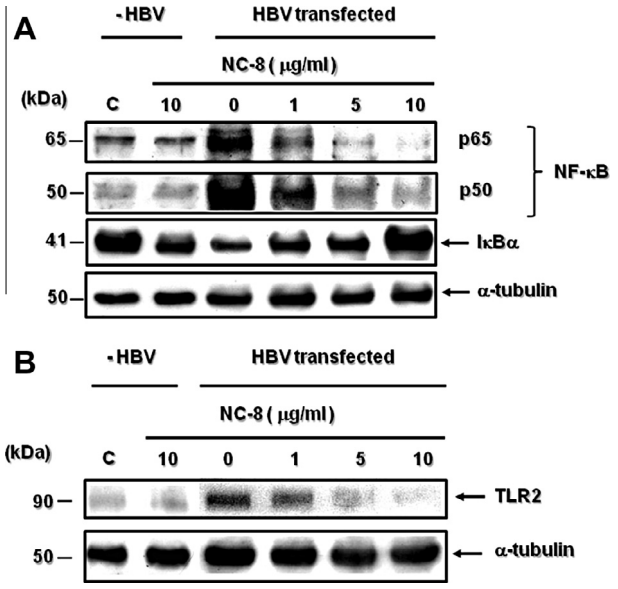


Fig. 5. Effects of NC-8 on NF-κB p65/p50, IκBα, and TLR2 protein expressions in Huh7 cells. Huh7 cells were seeded in 60-mm culture dishes and then transfected with or without the pHBV1.2 plasmid for 2 days. After treatment with or without NC-8 (**2**) (1–10 μg/ml) for another 2 days, cells were harvested, and total proteins were isolated for a Western blot analysis to determine expression levels of (A) NF-κB p65/p50, IκBα, or (B) TLR2 proteins. α-Tubulin protein expression served as the loading control. Data shown are representative of three sets of experiments.

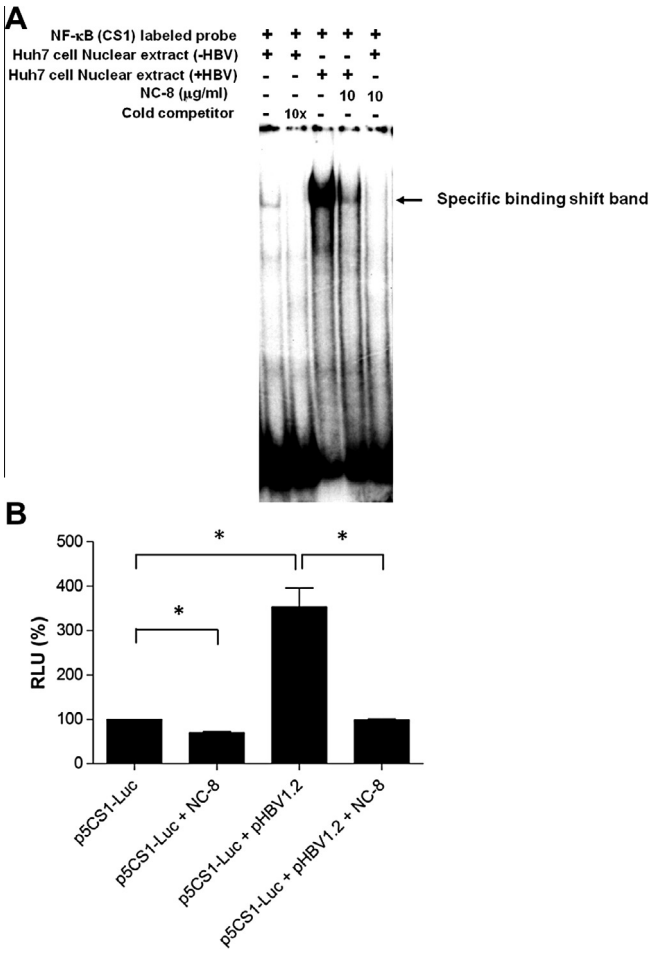


Fig. 6. Effects of NC-8 on (A) DNA-binding activity of NF-κB to the CS1 site and (B) CS1 site-containing promoter activity. (A) Huh7 cells were transfected with or without the pHBV1.2 plasmid for 2 days. After transfection, cells were treated with or without NC-8 (**2**) (10 μg/ml) for another 2 days. Cells were harvested, and nuclear extracts were prepared for a binding assay. (B) Huh7 cells were transfected with either the p5CS1-Luc promoter–reporter constructs together with or without the pHBV1.2 plasmid for 24 h and treated with or without NC-8 (**2**) for 2 days. Data are expressed as relative levels of luciferase activity compared to the NC-8 untreated control or pHBV1.2 non-transfected control. ($n = 3$) ($^*p < 0.05$).

without NC-8 (**2**) for 2 days and tested for luciferase activity. Results showed that CS1-containing promoter activity increased in HBV-expressing cells, but not in HBV-negative cells. Furthermore, treatment with NC-8 (**2**) significantly decreased CS1-containing promoter activity in HBV-expressing cells, but not in HBV-negative cells (Fig. 6B).

Discussion

Developing anti-HBV agents with novel modes of action to alleviate problems associated with currently approved drugs is a major challenge (Wakui et al., 2010). The current study found that NC-8 (**2**) exhibited unique anti-HBV activity by decreasing expressions of HBV DNA, RNA, and protein in Huh7 cells that expressed the HBV viral genome. Among them, four HBV viral transcripts decreased (Fig. 3A). NC-8 (**2**) potently eliminated expressions of three forms of the viral surface antigen and core protein in Huh7 cells (Fig. 3B). In addition, a Southern blot analysis indicated that NC-8 (**2**) significantly inhibited the production of encapsidated DNA intermediates of HBV particles (Fig. 2B). Therefore, it can be speculated that NC-8 (**2**) might suppress viral gene expression by modulating viral promoter activity. Further investigation demonstrated that activities of the four viral gene promoters (Core, X, S, and PreS) were abolished by NC-8 (**2**) in Huh7 cells that expressed the replicating HBV genome (Fig. 3C); in particular, viral S promoter activity was dramatically reduced. In contrast, this compound did not alter the viral promoter activity in HBV non-transfected cells (data not shown). These results suggest that HBV viral proteins may serve as auto-regulators of viral gene expression itself and disturb this action. In addition, NC-8 (**2**) seems to have a potential role in viral surface gene regulation. Previous studies showed that HBcAg and HBx were essential to HBV gene regulation (Kwon and Rho, 2002; Tang et al., 2005). To examine the possible mechanism by which NC-8 (**2**) affects the function of HBV in host cellular signaling pathways, transcriptional activities of three major signaling pathways, i.e., NF-κB, AP-1, and ISRE, were analyzed using a promoter-reporter assay. After transfection, NC-8 (**2**) specifically decreased NF-κB promoter activity, while somehow slightly increasing AP-1 and ISRE promoter activities in HBV-transfected and non-transfected cells (Fig. 4), suggesting that HBV infection might be required for the actions of these transcription factors on promoter activity-related genes and that NC-8 (**2**) could interfere this regulation. Previous reports showed that several DNA-binding elements for NF-κB are essential for HBV viral gene regulation. Even so, a study by Kwon and Rho (2002) clearly demonstrated that the HBC core protein (HBc) upregulates the HBV enhancer II/pregenomic promoter through the NF-κB binding site located upstream of the promoter. Therefore, the importance of NF-κB in HBV gene regulation is the main issue we addressed. To address the role of NF-κB in NC-8 (**2**)-regulated HBV viral promoter activity, the specific NF-κB activation inhibitor, BAY 11-7085, was used to examine this possibility. When Bay11-7085 was co-treated with NC-8 (**2**) in HBV-expressing cells after transfection, inhibitory effect on viral surface gene promoter activity was reversed to a non-treatment level (unpublished data), thus suggesting that the action of NC-8 (**2**) on HBV viral surface gene regulation might be mediated by NF-κB's activation.

Prior studies showed that the innate immune response serves as the first line of host defense against exogenous pathogen

infections, which is via recognition of pathogen-associated molecular patterns. In addition, TLRs are the primary pathogen pattern-recognition receptors involved in innate immunity. Activation of the TLR signaling pathway is induced by dimerization of receptors on the surfaces of cell membranes, which in turn triggers MyD88 activation, leading to the nuclear translocation and activation of NF-κB, and this regulates cellular gene expression and immune responses (Takeda and Akira, 2005). Among TLR members, TLR2 was found to be involved in the inhibitory effects of interleukin (IL)-1 on HBV replication (Thompson et al., 2009) that was shown to be expressed by Huh7 and HepG2 cells (Preiss et al., 2008). Thus, host TLR2/NF-κB signaling pathways inhibited by NC-8 (**2**) were investigated in an HBV-dependent manner. Results indicated that NC-8 (**2**) specifically reduced expressions of NF-κB and TLR2 proteins in HBV-expressing Huh7 cells, but not in HBV-negative Huh7 cells (Fig. 5). This implies that an inhibitory mode of NC-8 (**2**) on the NF-κB signaling pathway is through modulation of HBV gene expression. Previous studies showed that transcriptional activators like HBx (Doria et al., 1995), HBcAg (Kwon and Rho, 2002), a *C*-terminally truncated form of the middle-sized HBV surface antigen ($MHBs^t$) (Meyer et al., 1992), and HB large surface antigens (LHBs) (Hildt et al., 1996) are capable of activating NF-κB. Thus, this clearly suggests that NC-8 (**2**) specifically downregulates TLR2 and NF-κB expressions in HBV-infected hepatoma cells in addition to viral gene regulation. Lin et al. (2009) indicated that one NF-κB binding site (CS1, nt207 to 216) was identified in the HBV surface gene open reading frame (ORF) and proposed that this site can be upregulated by NF-κB. Thus, to examine the possible role of NC-8 (**2**) in binding NF-κB to the CS1 site, its inhibitory effect on the surface gene promoter mediated through this element was examined and analyzed by an EMSA. Results indicated that NC-8 (**2**) significantly decreased binding activity of NF-κB to the CS1 site in nuclear extracts of HBV-expressing cells (Fig. 6A). Furthermore, transfection of HBV into cells obviously increased CS1-containing promoter activity, and NC-8 (**2**) reduced this promoter activity (Fig. 6B). These results suggest that viral proteins can transactivate surface genes and are inhibited by NC-8 (**2**) through binding of NF-κB to the CS1 site. This study results thus suggested that HBV can utilize the TLR2/NF-κB signaling pathways as a major factor in viral gene regulation and virus propagation, whereas a discrepancy of anti-HBV activity on IL-1 arose in a comparative study by Preiss et al. (2008). They proposed that the inhibitory effect of IL-1 on HBV replication was mediated through activation of TLR2. However, elevated expressions of TLR2 and NF-κB in hepatocytes infected with HBV seemed to be critical to HBV gene upregulation and viral replication in our study. A possible explanation is that the early phase of an immediate HBV infection will trigger an innate immune response including TLR2 activation, lead to nuclear translocation of preexisting NF-κB, and inhibit HBV replication. In the long run, the production of viral proteins (i.e., Core and X proteins) is essential for upregulation of viral genes (Kwon and Rho, 2002; Tang et al., 2005). Unidentified cellular signaling factors are transactivated by viral proteins, induce novel synthesis of TLR2 and NF-κB, and then provide feedback that augments viral gene expression and viral replication. This suggests that HBV may take over host signaling pathways and switch this regulatory machinery toward its own gene expression and genome replication after infection, despite inhibitory effects of the TLR2/NF-κB signaling pathway on HBV in early stages of infection. Several issues still remain to be elucidated in the future, including how HBV regulates cellular c-Jun/c-Fos and Jak-Stat signaling pathways and finally activates AP-1 and ISRE in addition to NF-κB; how NF-κB is involved in regulating viral gene promoter activity other than the S gene; and how HBV switches the regulatory machinery and takes over cellular signaling pathways in the early or late phase of infection.

Conclusions

In summary, it's hypothesized that NC-8 (**2**) initially inhibits HBV RNA production by decreasing transcription of viral RNAs through downregulating levels of host TLR2 and NF-κB signaling factors required for transcription of viral genes. It was noted that downregulation of viral gene expression by NC-8 (**2**), especially of the HBV Core and X genes, disrupted feedback activation of HBV gene expression and further decreased viral RNA transcription. Indeed, this process is required for viral Core and X proteins to enhance the initiation of pregenomic 3.5-kb RNA (Hildt et al., 1996; Kwon and Rho, 2002). However, interactions of viral proteins with NF-κB and the contribution to S gene promoter activation are still unknown. The current study indicated that the mode of antiviral action of NC-8 (**2**) is distinct from that of typical HBV reverse-transcriptase/polymerase inhibitors and other inhibitors that activate the TLR2/NF-κB signaling pathway. Meanwhile, the action of NC-8 (**2**) on HBV infectious disease is not only via a unique signaling pathway (TLR2/NF-κB) but also other pathways like AP-1 and ISRE transcription factors. Mechanisms of AP-1 and ISRE exerted by NC-8 (**2**) to regulate HBV gene expression therefore warrant future investigation. To our knowledge, this is the first report of an isosteviol analog exerting an inhibitory effect on the hepatitis B virus.

Experimental

Chemistry

General

The instrumental analysis was described previously (Chang et al., 2008, 2009).

*Synthesis of NC-8 (**2**) and anti-HBV assay*

Isosteviol was obtained by acid hydrolysis of stevioside (Scheme 1) as described previously (Avent et al., 1990). Isocyanate (**1**) was synthesized as described previously (Militsina et al., 2007) and characterized by NMR and HRESIMS analyses. Subsequently, methylamine (4 mmole) and Et_3N were added to a solution of **1** (0.3 mmole) in DMF (5 ml). The reaction mixture was stirred overnight at room temperature. After addition of 1 N HCl, the resulting white precipitate was collected and crystallized from H_2O–MeOH to afford NC-8 (**2**) in 85% yield.

The same experimental procedure was adopted for the preparation of compounds **3**–**17**. The spectroscopic data of these compounds were given in Supplementary data. The anti-HBV activity was performed following procedures described previously (Cheng et al., 2011).

*4-Isocyanato-19-nor-ent-16-oxobeyeran (**1**).* ^{1}H NMR (500 MHz, C_5D_5N): δ 2.66 (dd, *J* = 18.5, 3.6 Hz, 1H), 1.79 (d, *J* = 18.5 Hz, 1H), 1.52–1.61 (m, 5H), 1.41–1.46 (m, 3H), 1.17–1.38 (m, 7H), 1.24 (s, 3H), 1.01 (s, 3H), 0.98 (d, *J* = 4.1 MHz, 1H), 0.89 (s, 3H), 0.84 (d, *J* = 11.6 MHz, 1H), 0.69 (td, *J* = 11.4, 3.3 MHz, 1H); ^{13}C NMR (125 MHz, C_5D_5N): δ 220.5, 122.5, 59.4, 54.7, 54.4, 54.3, 48.9, 48.5, 41.3, 40.2, 39.2, 38.3, 37.5, 37.2, 31.6, 20.2, 20.1, 20.0, 18.2, 14.0; HRESIMS *m*/*z* 316.2272 $[M+H]^+$ (calcd for $C_{20}H_{30}NO_2$, 316.2277).

*ent-16-Oxobeyeran-19-N-methylureido (NC-8) (**2**)*

White crystals; m.p. 251–253 °C; $[\alpha]^{26}_D$ -77.5 (*c* 0.55, CH_3OH); ^{1}H NMR (500 MHz, C_5D_5N): δ 6.75 (br s, 1H), 4.77 (s, 1H), 3.29 (br d, *J* = 13.4 MHz, 1H), 2.85 (d, *J* = 4.7 MHz, 3H), 2.47 (dd, *J* = 18.4, 3.5 MHz, 1H), 1.27–1.75 (m, 13H), 1.59 (s, 3H), 1.00–1.15 (m, 3H), 1.00 (s, 3H), 0.90 (d, *J* = 12.2 MHz, 1H), 0.87 (s, 3H), 0.77 (m, 1H); ^{13}C NMR (125 MHz, C_5D_5N): δ 220.9, 159.0, 56.7, 54.9,

54.9, 54.2, 48.7, 48.6, 41.0, 39.5, 39.3, 37.7, 37.3, 37.2, 28.3, 26.8, 20.2, 20.1, 19.6, 18.2, 15.1; HRESIMS *m/z* 347.2658 $[M+H]^+$ (calcd for $C_{21}H_{35}N_2O_2$, 347.2699).

Bioassays

Materials

Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM, and fetal bovine serum (FBS) were purchased from GIBCO/BRL (Gaithersburg, MD, USA). Enzyme immunoassay (EIA) kits for HBsAg and HBeAg were obtained from Johnson and Johnson (Skillman, NJ, USA). The anti-HBsAg and TLR2 antibodies for the immunoblot analysis were purchased from GeneTex (San Antonio, TX, USA). The anti-HBcAg, anti-NF-κB (p65/p50), and anti-IκBα antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-preS2 protein antibody of HBV was purchased from Chemicon (Millipore, Billerica, MA, USA). The DIG high prime DNA labeling and detection starter kit was purchased from Roche (Mannheim, Germany). Power SYBR Green PCR master mix was purchased from Applied Biosystem (Foster City, CA, USA). A dual-Luciferase reporter assay kit was purchased from Promega (Madison, WI, USA). The Trizol® total RNA isolation solution and Lipofectamine 2000 transfection reagent were purchased from Invitrogen (Carlsbad, CA, USA). The QIAquick Gel extraction kit was purchased from Qiagen (Valencia, CA, USA). pNF-κB-Luc, p-activator protein (AP)-1-Luc, and p-interferon-sensitive response element (ISRE)-Luc promoter-luciferase reporter constructs from Stratagene (La Jolla, CA, USA) were provided by Dr. Cheng-Wen Lin. Other reagents were purchased from GIBCO/BRL (Invitrogen, Carlsbad, CA, USA), Sigma (St. Louis, MO, USA), Bio-Rad (Hercules, CA, USA), Merck (Darmstadt, Germany), J.T. Baker (Mansfield, MA, USA), and Amresco (Solon, OH, USA). NC-8 was pre-dissolved in dimethyl sulfoxide (DMSO).

Cell culture and bioactivity assays

Huh7 cells were maintained in DMEM supplemented with heat inactivated 10% (v/v) FBS (GIBCO/BRL, Invitrogen) and 1% antibiotics at 37 °C in a humidified 5% CO_2 incubator as described previously (Lu et al., 1995). Initially, cells seeded at a density of 4×10^4/ml and grown in various cultural dishes at 80% of confluence were used in all experiments. In routine compound treatments, cells were treated with serially diluted concentrations of NC-8 (**2**) for 2–3 days. After treatment, levels of the viral surface antigen (HBsAg) and e antigen (HBeAg) in the culture media were measured by an EIA kit (Johnson and Johnson) according to the manufacturer's instructions. In another set of antiviral experiments, Huh7 cells were plated in 60-mm culture dishes, transfected with the pHBV1.2 plasmid, and treated with NC-8 (**2**) in 2% FBS for 48 h. Treated cells were subjected to total RNA, DNA, and protein extraction.

Cell viability assay

The cytotoxic effect of NC-8 (**2**) was determined by a CellTiter 96® AQ_{ueous} one solution cell proliferation assay kit (MTS) (Promega, Madison, WI, USA) to pinpoint the non-toxic test compound concentration in Huh7 cells. Briefly, Huh7 cells transfected with or without the pHBV1.2 plasmid for 48 h were treated with serial dilutions of NC-8 (**2**) ranging 2.5–160 μg/ml, and the toxicity of cells 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. Non-cytotoxic concentrations were used in the antiviral activity assays.

Sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blot analyses of intracellular viral antigens and cellular signaling proteins

Whole-cell lysates of Huh7 were prepared by washing cells in phosphate-buffered saline (PBS) three times and suspending in RIPA (10 mM Tris–HCl, 150 mM NaCl, 5 nM EDTA, 0.1% SDS, 1.0% Nonidet P-40, and 0.25% deoxycholate) buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 ng/ml aprotinin, and 5 ng/ml leupeptin protease inhibitors. Suspensions were subjected to shearing several times using a syringe with a 25-gauge needle, and then centrifuged at 14,000 rpm for 20 min at 4 °C. Cellular protein concentrations were determined by a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA), and 25 μg of protein from each sample was applied to SDS–PAGE. Proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Amersham, GE Healthcare, Buckinghamshire, UK) by electroblotting, and then blocked for 1 h with PBST (PBS buffer with 0.05% Tween-20) containing 4% nonfat milk. The immunoblot was incubated with a primary antibody followed by a horseradish peroxidase (HRP)-conjugated secondary antibody. The same membranes were then stripped and reprobed with an anti-α-tubulin antibody (Sigma) as the loading control.

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. Total RNA (5 μg) was denatured, separated on a 1.0% agarose gel (Amresco), and transferred to a positively charged Hybond-N^+ nylon membrane (Amersham Biosciences, Bucks, UK). 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 (Will et al., 1985), purified, labeled with a DIG high prime DNA labeling kit (Roche, Mannheim, Germany). 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).

Southern blot analysis of intracellular HBV-DNA synthesis

Encapsidated viral DNA was extracted from intracellular core particles as described by Pugh et al. (1988), fractionated on 1.0% agarose gels and transferred onto a Hybond N^+ membrane (GE Healthcare). HBV DNA was detected by a Southern blot analysis using the DIG-labeled full-length HBV probe.

Plasmid construction

All of the constructs were produced by standard recombinant DNA techniques (Sambrook et al., 1989). The pHBV1.2 plasmid containing a head-to-tail dimer of the HBV adw2 serotype was cloned into the *Eco*RI site of pGEM-7Zf (+) (Promega, Madison, WI, USA) (Blum et al., 1991), which was kindly provided by Dr. Cheng-Chan Lu (Department of Pathology, National Cheng-Kung University). To construct the four viral promoter-reporter plasmids, HBV genomic fragments corresponding to the Core (nt1636–1851), S (nt3114–220), PreS (nt2438–2855), and X (nt1071–1357) gene promoter regions were amplified by a polymerase chain reaction (PCR) using the pHBV1.2 plasmid as a template and were subsequently inserted into the *Sac*I/*Xho*I sites of the pGL4.17 luciferase-reporter expression vector (Promega). The reporter plasmid, p5CS1-Luc, which contains five copies of the CS1 site (nt207–216) of the HBV adw serotype genome, was constructed from the pGL4.17 expression vector. All of the DNA sequences were verified with appropriate restriction enzyme digestion and direct sequencing.

Transient transfection and luciferase assay

Huh7 cells were transfected with various plasmids using the Lipofectamine 2000 transfection reagent (Invitrogen). Briefly, cells

(at 4×10^4/ml) were plated in a 24-well culture plate and transfected in DMEM supplemented with 10% FBS for 24 h, washed with 1× PBS, incubated in DMEM supplemented with 2% FBS, and then treated with or without NC-8 (**2**) (10 μg/ml) for 2 days. The luciferase assay was performed according to the manufacturer's instructions (Promega). For promoter-reporter construct transfection, pHBV1.2 or a control vector (1 μg/ml/well) was co-transfected with the pRL-SV40 *Renilla* luciferase expression plasmid (0.02 μg/well) (Promega), which was used to normalize the basal level of luciferase activity.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

To detect DNA/protein-binding activity, nuclear extracts were prepared from Huh7 cells that were transfected with pHBV1.2 for 2 days and treated with or without NC-8 (**2**) (5 and 10 μg/ml) for another 2 days. For the EMSA, nuclear extracts (8 μg) per reaction were pre-incubated with or without a competitor and concomitantly mixed with 1 μg poly[dI–dC] (Sigma) and 1 μg salmon sperm DNA (Sigma) on ice for 20 min, and then incubated with a ^{32}P-labeled double-stranded DNA probe for 20 min at 30 °C. The mixture was separated on a 5% nondenatured polyacrylamide gel in 0.5 × TBE (Tris–borate–EDTA) buffer at 150 V for 90 min. The binding shift was detected from an autoradiograph. The oligonucleotide-probe for the DNA binding assay was the NF-κB CS1-binding site which corresponded to the HBV adw serotype (nt207–216), 5′-TACAGGCGGGGTTTTTCTTGTTG-3′ (Lin et al., 2009).

Statistical analysis

Data are expressed as the standard error (SE) of the mean from three independent experiments using GraphPad Prism (GraphPad Software, San Diego, CA, USA). Statistical analyses used Student's *t*-test. A p value of <0.05 was considered statistically significant.

Acknowledgements

This work was funded by grants from the Committee on Chinese Medicine and Pharmacy, Department of Health, Executive Yuan, Taiwan (CCMP101-RD-015), China Medical University (CMU99-N2-03-2 and CMU101-S-19), and the National Science Council of Taiwan (NSC98-2320-B038-011-MY3). The authors express heartfelt thanks to Dr. Cheng-Chan Lu for the pHBV1.2 and pHBV2 plasmids, and to Dr. Hsiao-Sheng Liu for the pGAPDH plasmid.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2013.12.014.

References

Avent, A.G., Hanson, J.R., de Oliveira, B.H., 1990. Hydrolysis of the diterpenoid glycoside, stevioside. Phytochemistry 29, 2712–2715.
Baltina, L.A., Flekhter, O.B., Nigmatullina, L.R., Boreko, E.I., Pavlova, N.I., Nikolaeva, S.N., Savinova, O.V., Tolstikov, G.A., 2003. Lupane triterpenes and derivatives with antiviral activity. Bioorg. Med. Chem. Lett. 13, 3549–3552.
Blum, H.E., Galun, E., Liang, T.J., von Weizsäcker, F., Wands, J.R., 1991. Naturally occurring missense mutation in the polymerase gene terminating hepatitis B virus replication. J. Virol. 65, 1836–1842.
Chang, S.F., Yang, L.M., Lo, C.H., Liaw, J.H., Wang, L.H., Lin, S.J., 2008. Microbial transformation of isosteviol and bioactivities against the glucocorticoid/androgen response elements. J. Nat. Prod. 71, 87–92, and references cited therein.
Chang, S.F., Chou, B.H., Yang, L.M., Hsu, F.L., Lin, W.K., Ho, Y., Lin, S.J., 2009. Microbial transformation of isosteviol oxime and the inhibitory effects on NF-B and AP-1 activation in LPS-stimulated macrophages. Bioorg. Med. Chem. 17, 6348–6353.
Cheng, S.Y., Wang, C.M., Hsu, Y.M., Huang, T.J., Chou, S.C., Lin, E.H., Chou, C.H., 2011. Oleanane-type triterpenoids from the leaves and twigs of *Fatsia polycarpa*. J. Nat. Prod. 74, 1744–1750.
Chisari, F.V., Filippi, P., Buras, J., McLachlan, A., Popper, H., Pinkert, C.A., Palmiter, R.D., Brinster, R.L., 1987. Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. Proc. Natl. Acad. Sci. USA 84, 6909–6913.
Chisari, F.V., 2000. Viruses, immunity, and cancer: Lessons from hepatitis B. Am. J. Pathol. 156, 1118–1132.
Doria, M., Klein, N., Lucito, R., Schneider, R.J., 1995. The hepatitis B virus HBx protein is a dual specificity cytoplasmic activator of Ras and nuclear activator of transcription factors. EMBO J. 14, 4747–4757.
Flekhter, O.B., Boreko, E.I., Nigmatullina, L.R., Tret'yakova, E.V., Pavlova, N.I., Baltina, L.A., Nikolaeva, S.N., Savinova, O.V., Galin, F.Z., Tolstikov, G.A., 2003. Synthesis and antiviral activity of ureides and carbamates of betulinic acid and its derivatives. Russ. J. Bioorg. Chem. 29, 594–600.
Geuns, J.M.C., 2003. Stevioside. Phytochemistry 64, 913–921.
Gish, R.G., 2005. Current treatment and future directions in the management of chronic hepatitis B viral infection. Clin. Liver Dis. 9, 541–565.
Gitlin, N., 1997. Hepatitis B: diagnosis, prevention, and treatment. Clin. Chem. 43, 1500–1506.
Hildt, E., Saher, G., Bruss, V., Hofschneider, P.H., 1996. The hepatitis B virus large surface protein (LHBs) is a transcriptional activator. Virology 225, 235–239.
Kwon, J.A., Rho, H.M., 2002. Hepatitis B viral core protein activates the hepatitis B viral enhancer II/pregenomic promoter through the nuclear factor B binding site. Biochem. Cell Biol. 80, 445–455.
Lin, Y.C., Hsu, E.C., Ting, L.P., 2009. Repression of hepatitis B viral gene expression by transcription factor nuclear factor-kappaB. Cell Microbiol. 11, 645–660.
Lu, C.C., Chen, M., Ou, J.H., Yen, T.S.B., 1995. Key role of a CCAAT element in regulating hepatitis B virus surface protein expression. Virology 206, 1155–1158.
Meyer, M., Caselmann, W.H., Schluter, V., Schreck, R., Hofschneider, P.H., Baeuerle, P.A., 1992. Hepatitis B virus transactivator MHBst: activation of NF-κB, selective inhibition by antioxidants and integral membrane localization. EMBO J. 11, 2991–3001.
Militsina, O.I., Strobykina, I.Y., Kovylyaeva, G.I., Bakaleinik, G.A., Kataev, V.E., Gubaidullin, A.T., Musin, R.Z., Tolstikov, A.G., 2007. Synthesis of Schiff bases on the basis of the isosteviol terpenoid. Russ. J. Gen. Chem. 77, 285–296.
Park, N.H., Song, I.H., Chung, Y.H., 2006. Chronic hepatitis B in hepatocarcinogenesis. Postgrad. Med. J. 82, 507–515.
Pramoolsinsup, C., 2002. Management of viral hepatitis B. J. Gastroen. Hepatol. 17, S125–S145.
Preiss, S., Thompson, A., Chen, X., Rodgers, S., Markovska, V., Desmond, P., Visvanathan, K., Li, K., Locarnini, S., Revill, P., 2008. Characterization of the innate immune signalling pathways in hepatocyte cell lines. J. Viral Hepat. 15, 888–900.
Pugh, J.C., Yaginuma, K., Koike, K., Summers, J., 1988. Duck hepatitis B virus (DHBV) particles produced by transient expression of DHBV DNA in a human hepatoma cell line are infectious in vitro. J. Virol. 62, 3513–3516.
Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory Press, New York.
Takasaki, M., Konoshima, T., Kozuka, M., Tokuda, H., Takayasu, J., Nishino, H., Miyakoshi, M., Mizutani, K., Lee, K.H., 2009. Cancer preventive agents. Part 8: Chemopreventive effects of stevioside and related compounds. Bioorg. Med. Chem. 17, 600–605.
Takeda, K., Akira, S., 2005. Toll-like receptors in innate immunity. Int. Immunol. 17, 1–14.
Tang, H., Delgermaa, L., Huang, F., Oishi, N., Liu, L., He, F., Zhao, L., Murakami, S., 2005. The transcriptional transactivation function of HBx protein is important for its augmentation role in hepatitis B virus replication. J. Virol. 79, 5548–5556.
Thompson, A.J., Colledge, D., Rodgers, S., Wilson, R., Revill, P., Desmond, P., Mansell, A., Visvanathan, K., Locarnini, S., 2009. Stimulation of the interleukin-1 receptor and Toll-like receptor 2 inhibits hepatitis B virus replication in hepatoma cell lines *in vitro*. Antivir. Ther. 14, 797–808.
Wakui, Y., Inoue, J., Ueno, Y., Fukushima, K., Kondo, Y., Kakazu, E., Obara, N., Kimura, O., Shimosegawa, T., 2010. Inhibitory effect on hepatitis B virus in vitro by a peroxisome proliferator-activated receptor-γ ligand, rosiglitazone. Biochhem. Biophy. Res. Commun. 396, 508–514.
Will, H., Cattaneo, R., Darai, G., Deinhardt, F., Schellekens, H., Schaller, H., 1985. Infectious hepatitis B virus from cloned DNA of known nucleotide sequence. Proc. Natl. Acad. Sci. USA 82, 891–895.
Zhu, S.L., Wu, Y., Liu, C.J., Wei, C.Y., Tao, J.C., Liu, H.M., 2013. Synthesis and in vitro cytotoxic activity evaluation of novel heterocycle bridged carbothioamide type isosteviol derivatives as antitumor agents. Bioorg. Med. Chem. Lett. 23, 1343–1346.