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Inhibitory effects of metachromin A on hepatitis B virus production via impairment of the viral promoter activity

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ABSTRACT

The currently available antiviral agents for chronic infection with hepatitis B virus (HBV) are pegylated interferon-α and nucleoside/nucleotide analogues, although it has been difficult to completely eliminate covalently closed circular DNA (cccDNA) from patients. To identify an antiviral compound targeting HBV core promoter, 15 terpenes originating from marine organisms were screened using a cell line expressing firefly luciferase under the control of the HBV core promoter. Metachromin A, which is a merosesquiterpene isolated from the marine sponge *Dactylospongia metachromia*, inhibited the viral promoter activity at the highest level among the tested compounds, and suppressed HBV production with an EC_{50} value of 0.8 μM regardless of interferon signaling and cytotoxicity. The analysis on the structure-activity relationship revealed that the hydroquinone moiety, and the double bonds at carbon numbers-5 and -9 in metachromin A are crucial for anti-HBV activity. Furthermore, metachromin A reduced the protein level but not the RNA level of hepatic nuclear factor 4α, which mainly upregulates the activities of enhancer I/X promoter and enhancer II/core promoter. These results suggest that metachromin A can inhibit HBV production via impairment of the viral promoter activity. Antiviral agents targeting the viral promoter may ameliorate HBV-related disorders regardless of remaining cccDNA.

1. Introduction

Hepatitis B virus (HBV), which is one of the causative agents of chronic liver diseases, including cirrhosis and hepatocellular carcinoma (HCC), affects more than 240 million people worldwide (Ott et al., 2012; Trepo et al., 2014). After entry of HBV into hepatocytes, the circular partially double-stranded viral DNA is converted into covalently closed circular DNA (cccDNA) in the nucleus. There are four promoters (core, preS1, preS2/S, and X promoters) and two enhancer regions (enhancer I and enhancer II, also referred to as the core upstream regulatory sequence: CURS) on cccDNA. Viral mRNAs with nucleotide lengths of 3.5, 2.4, 2.1, and 0.7 kb are transcribed by core, preS1, preS2 and X promoters, respectively. Enhancer I and II regulate the activities of these promoters. The 3.5-kb mRNA includes precore RNA (pcRNA) and pregenomic RNA (pgRNA). The pgRNA is translated into core and polymerase proteins, and provides a template for vial DNA synthesis via reverse transcription (Beck and Nassal, 2007; Quasdorff and Protzer, 2010; Seeger and Mason, 2000). Several host transcriptional factors regulate the core promoter, which consists of the basic core promoter and CURS. For example, specific region protein 1, chicken ovalbumin upstream promoter transcription factor 1, hepatocyte nuclear factor (HNF) 3, HNF4α, peroxisome proliferation activated receptor (PPAR) α, Forkhead Box O1, and retinoid X receptor α (RXRα) were reported to interact with the core promoter (Li and Ou, 2001; Shlomai and Shaul, 2009; Tang et al., 2001; Waris and Siddiqui, 2002; Yu and Mertz, 2003). Among these transcriptional factors, HNF4α is a

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crucial factor for regulation of pgRNA synthesis, which is regulated by binding of HNF4α to core promoter regulatory elements (Bar-Yishay et al., 2011; Kramvis and Kew, 1999; Tang et al., 2001).

Pegylated interferon-α (IFN-α) and oral nucleoside/nucleotide analogues (NAs) are employed for current antiviral therapies in chronic hepatitis B (CHB) patients. These antiviral agents are capable of preventing HBV production, resulting in amelioration of liver cirrhosis and HCC, and improvement of the survival rate of CHB patients (Trepo et al., 2014; Zoulim and Durantel, 2015). However, the serious issues with therapies using these drugs are occasional side effects and the emergence of drug-resistant viruses during long-term usage (Gish et al., 2012; Zoulim, 2011). In addition, it is difficult for these direct-acting antivirals to eradicate cccDNA and viral DNA fragments integrated into the host genome (Guo and Guo, 2015).

Terpenes, also referred to as terpenoids, are biosynthesized from five-carbon isoprene units and are classified according to the number of isoprene units as follows: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), and triterpenes (C_{30}). Meroterpenes are defined as a group of terpenoids with an additional chemical moiety such as an aromatic ring. Terpenes are employed for the treatment of human disease due to different biological activities. The anticancer drug Taxol® and antimalarial drug artemisinin are well known to be terpene-based drugs (Zhang and Demain, 2005). Although artesunate, which is a derivative of artemisinin, was reported to exhibit weak antiviral activity against HBV (Romero and Lavine, 1996), most of the other terpenes have not yet been tested for development of anti-HBV compounds.

Marine organisms are common as sources including terpenes, of which some exhibit antitumor, antiviral, and antimicrobial activities (Laport et al., 2009; Zhang and Demain, 2005). In this study, we screened 15 terpenes derived from marine organisms by HBV core promoter assay in order to find novel anti-HBV agents. Metachromin A, which is a merosesquiterpene isolated from the marine sponge *Dactylospongia metachromia*, inhibited HBV core promoter activity and HBV production at the highest level among the tested compounds. Furthermore, the structure-activity relationships and antiviral function of metachromin A were analyzed.

2. Materials and methods

2.1. Preparation of terpenes and derivatives

Terpenes used in this study were purified from marine organisms and are summarized in Supplemental Table 1. Metachromin A acetate was prepared by treatment with acetic anhydride and pyridine followed by conventional work-up to give an acetate. Metachromin A methyl ether was formed in methanol solution by adding TMS-diazomethane. Tetrahydrometachromin A was prepared by hydrogenating metachromin A under an H_2 atmosphere for 2 h in the presence of a catalytic amount of palladium on carbon. The crude product was separated to give one of the major stereoisomers by HPLC. The identity of all the derivatives was confirmed by ^{1}H NMR and ESIMS.

2.2. Cell lines

HepG2.2.15.7 and Hep38.7-Tet cell lines were kindly provided by Drs. K. Watashi and T. Wakita (National Institute of Infectious Diseases, Japan) (Ogura et al., 2014). The Huh7 GL4.18 CURS_BC_AeUS cell line (CURS-BCP reporter cell line) was established as described previously (Yamashita et al., 2015).

2.3. Evaluation of luciferase activity for HBV core promoter activity

CURS-BCP reporter cells were treated with several concentrations of each compound for 48 h and then harvested for estimation of luciferase activity as described previously (Yamashita et al., 2015). Cell viability was estimated by MTS assay using a Celltiter 96 aqueous one-solution cell proliferation assay kit (Promega, Madison, WI, USA).

2.4. Determination of anti-HBV activity based on production of HBV DNA

Hep38.7-Tet cells were cultured in the absence of tetracycline for 7 days and were used as HBV-producing cells. HepG2.2.15.7 or HBV-producing Hep38.7-Tet cells were treated with several concentrations of each compound and were then harvested 9 days post-treatment. The medium was exchanged with a fresh medium once every three days. The amount of supernatant HBV DNA was quantified using real-time quantitative PCR (qPCR) as described previously (Yamashita et al., 2015). Cell viability was estimated by MTS assay.

Table 1
Inhibitory effects of terpenes on HBV core promoter activity.

Sample No.	Name	Classification of terpenoids	Inhibition of HBV core promoter (EC_{50}[a]: μM)	Cytotoxicity (CC_{50}[b]: μM)
1	dendrolasin	sesquiterpene	>40.0	>40.0
2	heteronemin	sesterterpene	<5.0	<5.0
3	laurinterol	sesquiterpene	25.5	>40.0
4	metachromin A*	sesterterpene	9.2	>40.0
5	mycaperoxide A	sesterterpene	<5.0	<5.0
6	scalaradial	sesterterpene	29.3	>40.0
7	theonellin isothiocyanate	sesterterpene	39.0	>40.0
8	brianthein W	diterpene	29.7	>40.0
9	cacofuran B	diterpene	28.8	>40.0
10	ginamallene	diterpene	32.6	>40.0
11	kalihinol E*	diterpene	<5.0	>40.0
12	sarcophytol A	diterpene	34.1	>40.0
13	sarcophytoxide	diterpene	28.1	>40.0
14	spongia-13(16),14-dien-19-oic acid	diterpene	>40.0	>40.0
15	strongylophorine-3	meroterpene	>40.0	>40.0

Asterisk (*) indicates a hit compound.
[a] Fifty percent inhibitory concentration based on the reduction of HBV core promoter activity.
[b] Fifty percent cytotoxicity concentration based on the reduction of cell viability.