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Glycyrrhetic acid, but not glycyrrhizic acid, strengthened entecavir activity by promoting its subcellular distribution in the liver *via* efflux inhibition

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ABSTRACT

Entecavir (ETV) is a superior nucleoside analogue used to treat hepatitis B virus (HBV) infection. Although its advantages over other agents include low viral resistance and the elicitation of a sharp decrease in HBV DNA, adverse effects such as hepatic steatosis, hepatic damage and lactic acidosis have also been reported. Glycyrrhizin has long been used as hepato-protective medicine. The clinical combination of ETV plus glycyrrhizin in China displays better therapeutic effects and lower rates of liver damage. However, there is little evidence explaining the probable synergistic mechanism that exists between these two drugs from a pharmacokinetics view. Here, alterations in the plasma pharmacokinetics, tissue distribution, subcellular distribution, and *in vitro* and *in vivo* antiviral activity of ETV after combination with glycyrrhizic acid (GL) were analysed to determine the synergistic mechanisms of these two drugs. Specific efflux transporter membrane vesicles were also used to elucidate their interactions. The primary active GL metabolite, glycyrrhetic acid (GA), did not affect the plasma pharmacokinetics of ETV but promoted its accumulation in hepatocytes, increasing its distribution in the cytoplasm and nucleus and augmenting the antiviral efficiency of ETV. These synergistic actions were primarily due to the inhibitory effect of GA on MRP4 and BCRP, which transport ETV out of hepatocytes. In conclusion, GA interacted with ETV at cellular and subcellular levels in the liver through MRP4 and BCRP inhibition, which enhanced the antiviral activity of ETV. Our results partially explain the synergistic mechanism of ETV and GL from a pharmacokinetics view, providing more data to support the use of these compounds together in clinical HBV treatment.

1. Introduction

Approximately 400 million people worldwide suffer from hepatitis B virus (HBV) infection(Tang et al., 2014). Repeated and sustained HBV infection could cause chronic hepatitis and advanced-stage liver diseases, such as fibrosis, cirrhosis and even hepatocellular carcinoma(Levrero and Zucman-Rossi, 2016; Ringelhan and Protzer, 2015). In view of this, there is great interest in anti-HBV therapies, and the currently approved antiviral treatments include immune regulators (Kang et al., 2015) and nucleotide/nucleoside analogues (NAs)(Fung et al., 2011). Most NAs interfere with HBV polymerase to suppress viral DNA replication, and entecavir (ETV) is one of the most widely used first-line antiviral agents(Keating, 2011). Although it has advantages over other agents, such as low viral resistance and the induction of a sharp decrease in HBV DNA(Liang et al., 2012), adverse effects such as hepatic steatosis, hepatic damage and lactic acidosis have also been reported(Jun et al., 2013; Lange et al., 2009).

Therefore, complementary medicines should be considered to protect the liver from injury in HBV patients given NA therapy. Many traditional Chinese herbs such as schisandra (Jiang et al., 2015), silymarin (Vargas-Mendoza et al., 2014) and liquorice (Jung et al., 2016)

Abbreviations: HBV, hepatitis B virus; NAs, nucleotide/nucleoside analogues; ETV, entecavir; GL, glycyrrhizic acid; GA, glycyrrhetic acid; SD, Sprague–Dawley; MTR, mitoxantrone; CDCF, 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate; Rho123, rhodamine 123; HBSS, Hank's Balanced Salt Solution; qPCR, quantitative real-time PCR; ALT, alanine aminotransferase; AST, aspartate aminotransferase; TBIL, total bilirubin; DBIL, direct bilirubin; SLC transporter, solute carrier transporter; ABC transporter, ATP-binding cassette transporter; pg RNA, pregenomic RNA

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have long been used as hepato-protective agents. Glycyrrhizic acid (GL), extracted from liquorice, has been used in the clinical treatment of chronic hepatitis in China and Japan for > 20 years and exhibits anti-inflammatory (Marianecci et al., 2014), antiviral (Duan et al., 2015) and hepato-protective effects (Jeong et al., 2002; Manns et al., 2012). Furthermore, the synergistic action of GL with lamivudine (Matsuo et al., 2001; Tandon et al., 2001; Wakamatsu et al., 2007) in HBV treatment has been demonstrated. Therefore, it was hypothesized that the combination of GL and ETV might represent a new therapeutic strategy in HBV treatment. There is also evidence from clinical cases in China to support this new available treatment: a larger reduction of HBV DNA copies and a lower rate of liver damage were observed in patients treated with GL and ETV than in those treated with ETV alone (J. F. Xie et al., 2013; Zhang, 2013). However, most of the evidence has only demonstrated this beneficial effect of GL and ETV, and the specific mechanisms involved, especially from a pharmacokinetics viewpoint, remain unknown and require further investigation.

Traditional pharmacokinetics focuses primarily on plasma drug concentrations when evaluating drug-drug interactions. However, in many cases, the plasma drug concentrations are not changed, while the drug contents in organs or cells are altered after co-administration (Lau et al., 2015). As a result, we previously proposed that cellular pharmacokinetics, which is focused on drug metabolism and disposition in target cells or subcellular organelles, would allow for more accurate pharmacokinetics-pharmacodynamics correlation analyses (Zhang et al., 2012; Zhou et al., 2011). The accumulation of anti-HBV agents in liver tissue and their subcellular distribution in hepatocytes determines anti-HBV activity. Therefore, in the present study, on the basis of traditional pharmacokinetics evaluations, we further analysed ETV-GL interactions at the tissue, cellular and subcellular levels with *in vitro* and *in vivo* evidence, providing pharmacokinetics mechanisms to support this combination therapy for the treatment of HBV infection.

2. Material and methods

2.1. Reagents

Entecavir and diammonium glycyrrhizinate (purity > 98%) were obtained from Chia Tai Tianqing Pharmaceutical Group Co., Ltd. (Jiangsu, China). Glycyrrhetic acid (GA) and lamivudine (purity > 98%) were purchased from Tokyo Chemical Industry (Tokyo, Japan). Mitoxantrone (MTR), 5(6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDCF), Rhodamine 123 (Rho123), *N*-methyl quinine, Lucifer yellow, estradiol-17β-D-glucuronide, warfarin, and doxorubicin were purchased from Sigma (St. Louis, MO). LY335979, MK571 and KO143 were purchased from Selleckchem (Houston, Texas, USA). The Genomic DNA Extraction Kit and Premix Ex Taq for probe qPCR were purchased from Takara Bio Inc. (Otsu, Shiga, Japan). Deionized water was prepared by a Milli-Q system (Merck Millipore, Billerica, MA, USA). HPLC-grade methanol and acetonitrile were purchased from Merck (Darmstadt, Germany). Other regular chemicals used were of analytical grade and acquired from commercial sources.

2.2. Animals

Sprague-Dawley (SD) rats (200 ± 20 g) were supplied by SLAC Laboratory Animal Co., Ltd. (Shanghai, China). HBV(+) and HBV(−) mice were supplied by the Department of Laboratory Animal Science at Peking University Health Science Center (Beijing, China). All the animals were housed in a clean-grade environment at room temperature (22 ± 2 °C) with 50–60% relative humidity and an automatic day-night rhythm (12 h cycle). All animal experiments were approved by the Animal Ethics Committee of China Pharmaceutical University (Jiangsu, China). This study was carried out in strict accordance with the Guidelines for Animal Experimentation of this institution. All procedures were as humane as possible. Every effort was made to minimize animal pain, suffering and distress and to reduce the number of animals used.

2.3. Plasma pharmacokinetics studies

To explore the pharmacokinetics profiles of GL *in vivo*, five male SD rats received an oral dose of 100 mg kg^{-1} GL. Blood samples were collected at 0.08, 0.25, 1, 4, 6, 8, 10, 12, 16, 18, 20, and 24 h post-dosing *via* the jugular vein into heparinized tubes. Plasma samples were obtained after centrifugation and stored at − 20 °C.

To investigate whether oral GL administration would affect the pharmacokinetics of ETV after a single- or multiple-dosing regimen, twenty male SD rats were divided into four groups of 5 animals each.

For the single-dosing regimen, one group was intragastrically administered 100 mg kg^{-1} GL dissolved in normal saline, while the control group received only the vehicle (normal saline). Six hours later, 0.09 mg kg^{-1} ETV was given to the rats by intragastric (*i.g.*) administration. Blood samples were then collected *via* the jugular vein into heparinized tubes at 0.167, 0.5, 1, 2, 4, 8 and 24 h post-ETV dosing.

For the multiple-dosing regimens, rats were treated in the same manner as for single dosing each day for ten consecutive days. On the 10th day, blood samples were obtained *via* the jugular vein at the same time points as for single dosing after the last ETV administration.

To further elucidate whether the active metabolite GA would affect the ETV pharmacokinetics profile, ten rats were divided into two groups and received 0.09 mg kg^{-1} ETV (*i.g.*) with or without 20 mg kg^{-1} GA (*i.g.*). Blood samples were also obtained *via* the jugular vein at the same time points.

2.4. Tissue distribution studies

Twenty male SD rats were divided into four groups of 5 animals each. Two groups were treated with a single-dosing regimen, and the other two groups were treated with a multiple-dosing regimen. The drug treatments were the same as those described for the plasma pharmacokinetics studies. All the rats were sacrificed 2 h post-ETV administration (on the 1st day for single dosing and 10th day for multiple dosing). Blood and tissues, including the brain, kidney, liver, heart, stomach and intestine, were collected. Plasma was obtained by centrifugation. The plasma and tissues were stored at − 20 °C until analysis.

2.5. Rat primary hepatocyte culture

Rat primary hepatocytes were isolated by two-step collagenase perfusion with isotonic Percoll purification as described previously (Guo et al., 2014). The obtained viable hepatocytes were seeded in rat tail collagen-coated tissue culture plates at a concentration of 2.5×10^5 cells ml^{-1}. The cells were cultured in DMEM with 5% foetal bovine serum (FBS), 50 U ml^{-1} penicillin/streptomycin, 4 mg l^{-1} insulin and 1 μM dexamethasone at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.6. HBV-infected and non-infected HepG2 cell culture

The human hepatoma cell line HepG2 and the HBV-infected HepG2 cell line HepG2.2.15 were obtained from the China Center for Type Culture Collection in Wuhan (Hubei, China). Both cell lines were grown in DMEM supplemented with 10% FBS and 100 U ml^{-1} penicillin and streptomycin (Life Technologies, Carlsbad, CA, USA) at 37 °C with 5%