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Research Article

Kushenin induces the apoptosis of HCV-infected cells by blocking the PI3K-Akt-mTOR pathway via inhibiting NS5A

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ABSTRACT

With the increased burden induced by HCV, there is an urgent need to develop better-tolerated agents with good safety. In this study, we evaluated the anti-HCV capability of kushenin, as well as the possible mechanism to Huh7.5-HCV cells. The results demonstrated that kushenin significantly inhibited the HCV-RNA level. Similarly, the expression of HCV-specific protein NS5A was also decreased. Molecular docking results displayed that kushenin bonded well to the active pockets of HCV NS5A, further confirming the effects of kushenin on HCV replication. Coimmunoprecipitation assay determined that kushenin suppressed the interaction between PI3K and NS5A in HCV-replicon cells. Furthermore, kushenin exerted an obviously induced function on HCV-replicon cells apoptosis by inhibiting PI3K-Akt-mTOR pathway, which could be ameliorated by the specific activator IGF-1 addition. Taken together, kushenin possesses the ability to inhibit HCV replication, and contributes to the increased apoptosis of HCV-infected cells by blocking the PI3K-Akt-mTOR pathway via inhibiting NS5A. Our results provide important evidence for a better understanding of the pathogenesis of HCV infection, and suggest that kushenin has the potential to treat HCV disease.

1. Introduction

Viral hepatitis C (HC), a widespread viral disease caused by the hepatitis C virus (HCV), affects more than 170 million individuals worldwide [1]. By 2030, HC mortality rates are expected to exceed those of HIV/AIDS in many countries [2]. Meanwhile, HCV is one of the main causes of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [3,4]. Factors, referred to as host factors, such as co-infections and exposure to toxic agents, can contribute to this progression [5]. On the other hand, the viral genotype is also significant to HC deterioration [6]. Genotype 2 has been shown to be sensitive to various direct-acting anti-virals (DAAs), while genotype 3 was found to be more difficult to treat [7].

So far, only a small number of patients treated with interferon alpha (INF-α)-based medication have received therapeutic benefits because of the acute rejection reactions to INF-α [8,9]. In addition, vaccination is considered to be the most effective way to control the spread of the virus. Unfortunately, no satisfactory vaccines targeting HCV are available presently. In 2005, Melhem et al. suggested that the pathogenesis of HCV infection was associated with a defective host antiviral immune response and intrahepatic oxidative stress. The combination of antiviral and antioxidative therapies may enhance the overall response rate of these patients [10]. There are numerous additional agents and viral targets in various stages of clinical and preclinical testing, such as ribavirin and siRNA [11–14]. However, there is still a great and urgent need to develop more effective therapeutic options or better-tolerated agents with good safety profiles to improve HCV treatment outcomes.

According to the results of the World Health Organization's (WHO) investigation, approximately 70–80% of the world's population uses complementary and alternative medicine (CAM) modalities, especially in China, where there is an abundance of traditional herbal medicine resources [15]. Kushenin, a mixed alkaloid consisting of oxymatrine with a little oxysophocarpine, is extracted from the root of Chinese herbal medicine Sophora flavescens [16]. Its pharmacological functions include acting as an antivirus, regulating immune reactions, and having anti-fibrosis, anti-arrhythmia, anti-inflammation, and antibacterial properties, among other important qualities [16–18]. The effects and underlying mechanisms of kushenin on HCV remain undefined. The long-term progress of viral hepatitis using inhibition by oxymatrine, which is similar to the main component of kushenin, may provide new insights into the application of kushenin in the control of HCV. Oxymatrine, an alkaloid extracted from Sophora

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alopecuraides L, has also been shown to effectively and safely suppress HBV replication [19,20]. In 2001, Chen further identified the inhibitory effect of oxymatrine on the hepatitis C virus in vitro [21]. Based on these results, in the present study, we investigated whether the administration of kushenin had some effects on inhibiting HCV.

2. Materials and methods

2.1. Cell lines and culture

Human HCC cells lines Huh7.5.1 were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS, Gibco, Rockville, MD), 100 U/mL penicillin, 100 mg/mL streptomycin and 0.5 mg/mL G418 (Invitrogen, Carlsbad, CA) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. The establishment of Huh7.5.1-HCV cells

Huh7.5.1 cells expressing the HCV genotype 2a subgenomic replicon containing Renilla luciferase reporter (J6/JFH1 HCVcc) was established as Lindenbach et al. previously described [22]. Briefly, plasmid pFLJ6/JFH1, which contains the full-length genomic cDNA of HCV J6 and JFH-1, was linearized. Then, an in vitro MEGA script kit (Promega, Wisconsin, USA) was applied for the transcription, with the prepared plasmid pFLJ6/JFH1 serving as the template. The in vitro-transcribed RNA was delivered to Huh7.5.1 cells by electroporation. After 8–18 days of transfection, viral stocks were obtained from culture supernatants after storage at −80 °C before immunostaining for viral stock titer. When HCVcc-infected cells were washed and fixed with cold methanol, anti-HCV-positive sera were probed. After washing and probing with FITC-conjugated anti-human IgG (Jackson ImmunoResearch, UK), the cells were labeled with DAPI. The fluorescence microscope (IX81; Olympus, Tokyo, Japan) was applied to obtain the images. Stained foci were counted in quadruplicate wells, as well as the virus titer of focus-forming units (ffu)/mL. Finally, HCVcc supernatant was added to Huh7.5.1 cells for 5 h, which were then supplied with fresh medium after washing.

2.3. Cell viability assay

Cell viability was determined by a colorimetric MTT assay. Briefly, the cells were first seeded in 96-well plates (3000 cells/200 μL). Subsequently, they were treated with different concentrations (0 μg/mL, 10 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL, 100 μg/mL and 200 μg/mL) of kushenin (Fig. 1a) (Chia tai Tianqing Pharmaceutical Group Co., Ltd., Jiangsu, China) for various lengths of time (12 h, 24 h, 48 h and 96 h). Then, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO) solution (final concentration 0.5 mg/mL) was added and the samples were incubated for another 4 h at 37 °C. The medium was then removed and 200 μL dimethyl sulfoxide (DMSO) was added for 15 min to dissolve the formazan dye crystals. Absorbance at 570 nm was finally measured using a microplate reader with a reference wavelength of 630 nm. Results were expressed as the

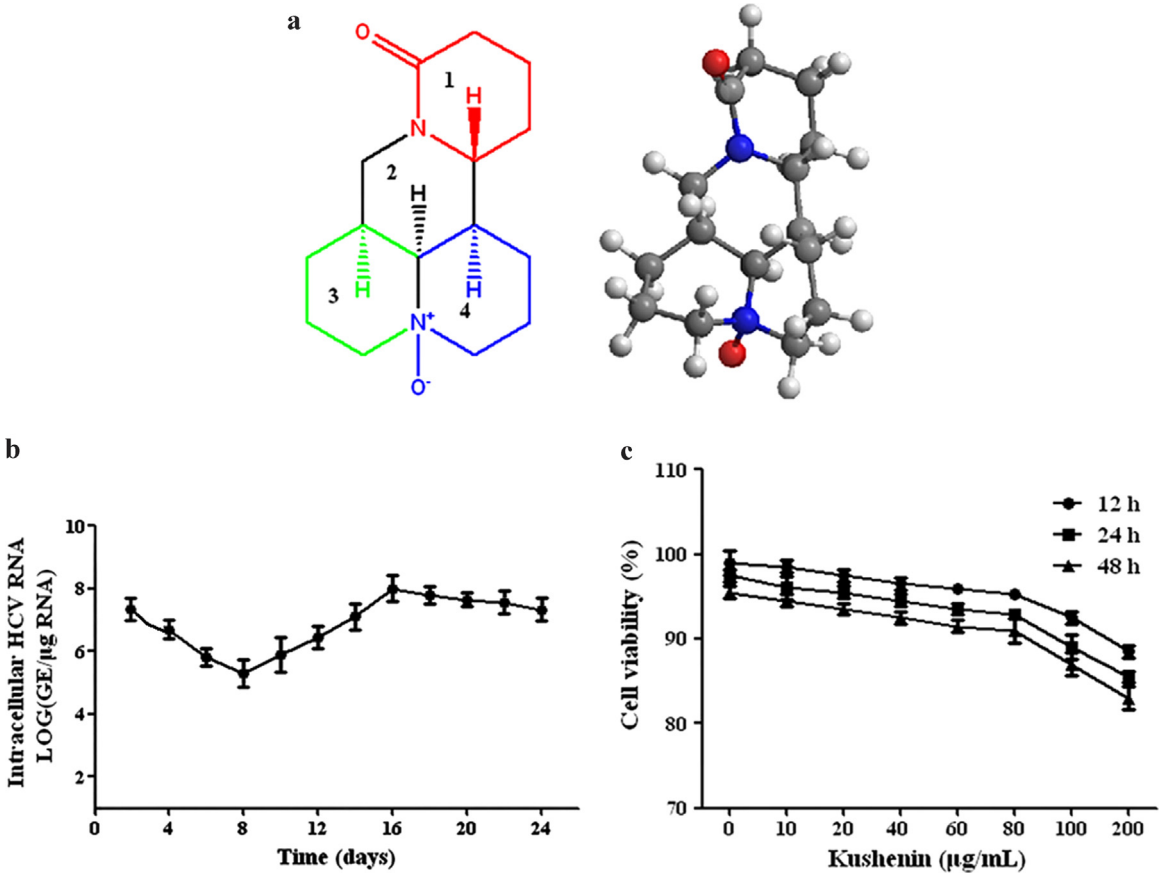

Fig. 1. Production of infectious HCV and the cytotoxicity of kushenin to Huh7.5-HCV cells. (a) The biochemical structure and spacial conformation of kushenin. (b) Transfected cells were harvested at the indicated time points after transfection. qRT-PCR was applied to evaluate the production of infectious HCV after transfection of genomic J6/JFH1 RNA. The results were displayed as genome equivalents (GE)/μg total RNA. (c) Cells were treated with varying concentrations of kushenin (0 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL, 100 μg/mL) for different time periods (12 h, 24 h, 48 h). Cell viability was measured using an MTT assay.