Research Article

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# Saikosaponin b2 is a naturally occurring terpenoid that efficiently inhibits hepatitis C virus entry

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**Background & Aims**: A vaccine against hepatitis C virus (HCV) is unavailable and cost-effective antivirals that prevent HCV infection and re-infection, such as in the transplant setting, do not exist. In a search for novel and economical prophylactic agents, we examined the antiviral activity of saikosaponins (SSa, SSb2, SSc, and SSd) from *Bupleurum kaoi* root (BK) as entry inhibitors against HCV infection.

**Methods**: Infectious HCV culture systems were used to examine the effect of saikosaponins on the complete virus life cycle (entry, RNA replication/translation, and particle production). Antiviral activity against various HCV genotypes, clinical isolates, and infection of primary human hepatocytes were also evaluated.

**Results**: BK and the saikosaponins potently inhibited HCV infection at non-cytotoxic concentrations. These natural agents targeted early steps of the viral life cycle, while leaving replication/translation, egress, and spread relatively unaffected. In particular, we identified SSb2 as an efficient inhibitor of early HCV entry, including neutralization of virus particles, preventing viral attachment, and inhibiting viral entry/fusion. Binding analysis, using soluble viral glycoproteins, demonstrated that SSb2 acted on HCV E2. Moreover, SSb2 inhibited infection by several genotypic strains and prevented binding of serum-derived HCV onto hepatoma cells. Finally, treatment with the compound blocked HCV infection of primary human hepatocytes.

**Conclusions**: Due to its potency, SSb2 may be of value for development as an antagonist of HCV entry and could be explored as prophylactic treatment during the course of liver transplantation.


Keywords: Hepatitis C virus; Virus entry; Inhibitor; Antiviral; Saikosaponin; Liver transplant.

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*Abbreviations:* AST, aspartate aminotransferase; ALT, alanine aminotransferase; $CC_{50}$, 50% cytotoxic concentration; DMSO, dimethyl sulfoxide; $EC_{50}$, 50% effective concentration; HAV, hepatitis A virus; HBV, hepatitis B virus; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; MOI, multiplicity of infection; PBS, phosphate buffered saline; PFA, paraformaldehyde; qRT-PCR, quantitative real-time PCR; RLU, relative light units; VSV, vesicular stomatitis virus.

## Introduction

Persistent infections by HCV can lead to cirrhosis and hepatocellular carcinoma (HCC), making hepatitis C the most common indication for liver transplantation in Western countries [1]. Without a preventive vaccine, infected individuals have relied on standard treatment, consisting of pegylated (Peg)-interferon (IFN)-α in combination with ribavirin (RBV) for over a decade. This regimen is expensive and often associated with poor antiviral response and unwanted sides-effects [2]. Alternatively, liver transplantation is compounded by issues of donor shortage, graft rejection risk, recurrent infections, and absence of adequate therapy against reinfection [3]. While novel protease inhibitors such as boceprevir and telaprevir have been approved for treatment against hepatitis C, these drugs lack pan-genotype activity and are associated with drug toxicity and development of resistant mutants [4,5]. Furthermore, new improved second generation drugs, such as simeprevir, sofosbuvir, and daclatasvir, promise to be extremely expensive for managing chronic infections, making accessibility of therapy a potential difficulty. A highly effective combination treatment would likely be required for the future management of HCV infections and entry inhibitors could play an important role. Thus far, no entry inhibitor has been licensed for prophylactic treatment of hepatitis C and continuous

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efforts in developing cost-effective anti-HCV therapeutics are essential to minimize the morbidity associated with the disease.

HCV is an enveloped flavivirus that possesses a positive RNA genome. The hepatotropic virus gains entry into hepatocytes through interaction of its glycoproteins with entry factors and receptors, including glycosaminoglycans, low-density lipoprotein receptor, scavenger receptor class B type I, the tetraspanin family protein member CD81, and tight junction proteins including claudins (CLDN-1, 6, and 9), and occludin [6]. Translation of the viral RNA generates a single polyprotein that is processed by viral and host proteases into 10 different proteins. These include: the viral core and envelope glycoprotein 1 (E1) and 2 (E2), which make up the structural proteins; the ion channel p7; and the non-structural (NS) proteins, which consist of NS2, NS3, NS4A, NS4B, NS5A, and NS5B [7]. The error-prone viral polymerase NS5B [8] has made HCV a genetically variable pathogen with multiple genotypes (1–7) and an array of subtypes (a, b, etc.).

*Bupleurum* spp. roots (Radix Bupleuri) are frequently used as herbal treatments for liver diseases in Eastern Asia. Their pharmacology and chemical composition have been extensively studied, and among its bioactive molecules are the saikosaponins (major series: SSa, b2, c, and d) [9]. These phytochemicals are glycosides, bearing a pentacyclic triterpene structure and resemblance to steroid hormones. The saikosaponins possess a variety of biological effects. Most notable are their liver-protective functions against liver fibrosis [10], HBV infection [11], and hepatocarcinogenesis [12]. However, it is unclear whether saikosaponins and the *Bupleurum* spp. root itself possess antiviral activity against HCV.

Subgenomic replicons [13] and JFH-1 based infectious culture systems [14] have provided powerful tools to dissect the HCV life cycle and develop drugs that target specific steps of the viral infection. Using these tools, we demonstrate that methanolic extracts from the roots of *Bupleurum kaoi*, and its purified saikosaponin components SSa, SSb2, SSc, and SSd, inhibit HCV infection. We identified SSb2 as the most effective anti-HCV agent and showed that it interacts with HCV particles and blocks virus entry of the host cell.

## Materials and methods

### *Cell culture and reagents*

Human hepatoma HuH7 cells and their derivatives HuH7.5 and S29 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GIBCO-Invitrogen; Carlsbad, CA, USA), supplemented with 10% foetal bovine serum (FBS; GIBCO-Invitrogen), 50 μg/ml gentamicin (GIBCO-Invitrogen), and 0.5 μg/ml amphotericin B (GIBCO-Invitrogen). The sbJFH1-B2 replicon cells are a HuH7 cell line, carrying subgenomic HCV JFH1 (genotype 2a) RNA, and were maintained in the above described media supplemented with 1 mg/ml of G418 (GIBCO-Invitrogen). Recombinant human IFN-α was purchased from Sigma (St. Louis, MO, USA; used at 800 IU/ml) and soluble HCV glycoprotein E2 (sE2; genotype 1a) was obtained from AUSTRAL Biologicals (San Ramon, CA, USA).

### *Plasmids*

The subgenomic reporter HCV plasmid pSGR-Luc-JFH1 and its NS5B polymerase-inactive control, pSGR-Luc-JFH1/GND [15], were gifts from John McLaughlin. The full-length genotype 2a reporter HCV genomes Jc1FLAG2(p7-nsGluc2A) and J6/JFH(p7-Rluc2A) have been recently described [16,17] and were provided by Charles M. Rice. The assembly-defective J6/JFH(p7-Rluc2A)ΔE1E2 contains an in-frame deletion of the envelope proteins [17]. The constructs J8/JFH1 (genotype 2b), S52/JFH1 (genotype 3a), and QC69/JFH1 (genotype 7a) were provided by Jens Bukh. These chimeras encode the viral core, E1, and E2, which make up the structural proteins, as well as p7, and NS2 sequences from genotype 2b, 3a, and 7a, and are built on a JFH1 backbone [18,19].

### *In vitro transcription, electroporation and production of infectious HCV*

*In vitro* transcription and electroporation of HCV RNA genomes into recipient cells, as well as production of infectious HCV, were performed as previously described [14,16,20]. The cell-culture derived HCV (HCVcc) particles, produced from run-off transcripts of Jc1FLAG2(p7-nsGluc2A), were designated as "Jc1-GLuc" virus in this study.

Additional experimental procedures are provided in the Supplementary materials and methods section.

## Results

### *Methanolic extract of Bupleurum kaoi roots (BK) and the saikosaponins*

To identify novel HCV antivirals, we performed a dose-response analysis of HuH7.5 cells infected with *Gaussia* luciferase reporter viruses (Jc1-GLuc) in the presence of a saponin-rich methanolic extract of *Bupleurum kaoi* roots (BK; Supplementary Fig. 1) or its associated saikosaponins SSa, SSb2, SSc, and SSd. BK, as well as the saikosaponins, inhibited HCV infections at non-cytotoxic concentrations (Fig. 1A). The effective concentrations ($EC_{50}$) are listed in Fig. 1B. Among the samples tested, SSb2 was the most selective (toxic dose/effective dose), yielding the highest SI score, followed by SSa, BK, SSd, and SSc (Fig. 1B). The most effective concentrations of BK and the saikosaponins were chosen for the remainder of the study: BK = 50 μg/ml, SSa = 8 μM, SSb2 = 50 μM, SSc = 200 μM, and SSd = 2 μM,

### *BK and the saikosaponins inhibit the early stages of HCV infection*

To better understand the antiviral effects of BK and the saikosaponins against HCV, we examined their antiviral activity in a series of synchronized infection assays. Wells containing HuH7.5 cells were treated with the test compounds at different times during an infection with the Jc1-GLuc virus and then analysed for luciferase activity. The anti-HCV effects due to BK, SSa, SSb2, and SSd were most effective when they were added together with the virus, compared to pretreatment of the naïve monolayer or treatment of previously infected cells (Fig. 2A). This observation suggested that BK and most of the saikosaponins likely target HCV during the initial stages of infection. As a control, IFN-α, which acts by inducing the cellular antiviral response, was effective throughout the time-course. To further explore their inhibitory activity on HCV entry, the effect of BK and the saikosaponins on the free virus particles, as well as their influence on viral attachment and entry/fusion was analysed. Test compounds were added to Jc1-GLuc virus or cells prior to infection, or to virus adsorbed to cells prior to entry/fusion and shifting the incubation temperatures between 4 °C (where the virus binds, with no entry/fusion) and 37 °C (entry/fusion occurs). SSb2 was able to block infection by neutralizing free virus particles and abolishing viral attachment and entry/fusion (Fig. 2B). In comparison, BK and SSa appeared to be most effective during the entry/fusion event.