ORIGINAL ARTICLE

Antiviral effect of saikosaponin B2 in combination with daclatasvir on NS5A resistance-associated substitutions of hepatitis C virus

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Abstract

Background: Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. The rapid progress in the development of direct-acting antivirals[1] has greatly elevated the cure rate to ≥95% in recent years. However, the high cost of treatment is not affordable to patients in some countries, necessitating the development of less expensive treatment.

Methods: We adopted a cell culture-derived HCV system to screen a library of the pure compounds extracted from herbs deposited in the chemical bank of the National Research Institute of Chinese Medicine, Taiwan.

Results: We found that saikosaponin B2 inhibited viral entry, replication, and translation. Saikosaponin B2 is a plant glycoside and a component of *xiao-chai-hu-tang*, a traditional Chinese herbal medicine extracted from the roots of *Bupleurum falcatum*. It also inhibited daclatasvir-resistant mutant strains of HCV, especially in combination with daclatasvir.

Conclusion: Our results may aid the development of a new combination therapy useful for patients with HCV who are intolerant or refractory to the currently available medications, including pegylated interferon and direct-acting antiviral agents.

Keywords: Daclatasvir; HCV; NS5A; Resistance-associated substitution; Saikosaponin

1. INTRODUCTION

Hepatitis C virus (HCV), a positive-stranded RNA virus of the *Hepacivirus* genus within the *Flaviviridae* family,[1,2] is a major cause of chronic hepatitis, which may progress to liver cirrhosis and hepatocellular carcinoma.[3] HCV was first identified as the cause of non-A, non-B hepatitis in 1989.[4] It infects approximately 200 million people worldwide annually.[5] Its 9.5-kb genome encodes a large polyprotein of approximately 3000 amino acids that is processed by host and viral proteases into at least 10 individual proteins: core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B.[6,7] The HCV life cycle starts from the binding of HCV particles to receptors on hepatocytes, such as CD81, SR-BI (scavenger receptor class B type I), and low-density lipoprotein receptor. HCV RNA replication and translation occur in the cytoplasm and endoplasmic reticulum, respectively, followed by assembly and release, which are related to the very-low-density lipoprotein biosynthesis.[8,9]

Although several novel direct-acting antiviral agents (DAAs)[1] are now available, the cost of treatment remains too high to be affordable in some countries. Herbal alternatives can be more affordable. The herbal derivative silymarin has been used for various chronic hepatitis.[10] In addition to silymarin, a number of compounds extracted from herbal plants have been used to treat HCV infection, including epigallocatechin-3-gallate (EGCG),[11] ladanein-BJ486K,[12] naringenin,[13] quercetin,[14] and apigenin.[15] In East Asia, *Bupleurum* spp. roots (Radix Bupleuri) have been widely used for treating liver diseases. Their chemical compositions have been extensively studied, and the bioactive molecules are named saikosaponins; among them, saikosaponin A, B2, C, and D are commonly used in clinical practice.[16] These phytochemicals are glycosides with a pentacyclic triterpene and possess various biological effects, notably including liver protection against fibrosis,[17] chronic hepatitis B,[18] chronic hepatitis C,[19] and hepatocarcinogenesis.[20]

Advances in the cell culture-derived HCV (HCVcc) system have allowed researchers to assess the direct effects of herbal medicines on the HCV life cycle, including RNA replication, virion production, and infectivity.[21–24] Using an HCVcc system, in cooperation with National Research Institute of Chinese Medicine (NRICM) in Taiwan, we screened the pure compounds extracted from Chinese herbs deposited in the chemical bank to

identify the compounds inhibiting HCV infection. In this study, we identified saikosaponin B2 in relation to inhibition of viral entry, replication, and translation. Saikosaponin B2 also inhibited daclatasvir-resistant mutant strains of HCV, especially in combination with daclatasvir. Our results may help design new combination therapy to help patients who are intolerant to the side effects of pegylated interferon or refractory to current DAAs.

2. METHODS

2.1. Chemicals and reagents
The herbal pure compound saikosaponin B2 was provided by Yun-Lian Li (NRICM, Taipei, Taiwan). Daclatasvir sodium (purity 99.47%) was purchased from Mesochem Co. Ltd. (Beijing, China). All chemicals were resuspended in dimethylsulfoxide (DMSO).

2.2. Cell culture
Huh-7.5.1 and 293T cells were grown in Dulbecco's modified minimal essential medium (Life Technologies) supplemented with 2 mM L-glutamine, nonessential amino acids, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal calf serum. Huh-7.5.1 cells stably expressing HCV genotype 1b Con1 replicon or 2a JFH1 replicon were established through transfection with the pFK-I$_{389}$neo-ubi-luc-NS3-3′/5.1 or pSGR-JFH1 RNA, respectively, followed by G418 selection.

2.3. HCV constructs
The constructs of HCV subgenomic Con1 (genotype 1b) and JFH1 (genotype 2a) replicons contained the firefly luciferase gene—pFK-I$_{389}$luc-NS3-3′/5.1[25] (University of Heidelberg, Heidelberg, Germany) and pSGR-Luc-JFH1[26] (MRC Virology Unit, Institute of Virology, Glasgow, UK), respectively.

J6/JFH(p7-Rluc2A) is a monocistronic reporter virus encoding the HCV polyprotein up to nucleotide 2783 (end of p7), *Renilla* luciferase, foot-and-mouth disease virus autoproteolytic peptide 2A, and the remainder of the HCV polyprotein coding sequence starting with amino acid two of NS2.[27] It is based on the J6/JFH genome with the intragenotypic break point between NS2 and NS3.[22]

We used pHCV-F.Luc-3′-UTR,[28] containing the firefly luciferase gene driven by the HCV internal ribosome entry site,[29] to investigate the effect of saikosaponin B2 on HCV translation.

To prepare RASs[30] of HCVcc (NS5A-L31M, Y93H, or L31M/Y93H) conferring resistance to daclatasvir, NS5A mutations were introduced into the J6/JFH (p7Rluc-2A) template through polymerase chain reaction (PCR) using specific primers corresponding to the *NS5A* gene using QuikChange Lightning Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primer sequence was 5′-GGG AGG CCG GGC ATC TTG GGG AAC AAT-3′, 5′-ATT GTT CCC CAA GAT GCC CGG CCT CCC-3′ (for L31M), 5′-CCT TTC CTA TCA ATT GCC ACA CGG AGG GCC-3′, and 5′-CTG GCC CTC CGT GTG GCA ATT GAT AGG AAA GG-3′ (for Y93H).

2.4. In vitro transcription, HCV RNA transfection, and infectious HCV production
In vitro RNA transcripts were generated from the linearized plasmids, J6/JFH (p7Rluc-2A), pFK-I389neo-ubi-luc-NS3-3′/5.1, pSGR-JFH1, pSGR-Luc-JFH1, or pHCV-F.Luc-3′-UTR using a MEGAscript T7 kit (Ambion, Austin, TX, USA) according to the manufacturer's instruction. The transcribed RNAs were transfected into Huh-7.5.1 cells for infectious HCV production[31] or for studying HCV replication and polyprotein translation, respectively.

2.5. Herbal drug screening
Huh-7.5.1 cells were seeded in 96-well plates at a density of 1 × 10⁴ cells per well and incubated in complete growth medium containing 0.5% DMSO with or without the indicated concentrations (42 μg/μL) of a pure herbal compound for 1 hour, followed by infection with J6/JFH (p7-Rluc2A) (multiplicity of infection of 0.1). Cultures were incubated for an additional 48 hours and then subjected to luciferase assay according to the manufacturer's instructions (Promega, Madison, WI, USA).

2.6. Cell viability assay
Huh-7.5.1 cells were processed in 96-well plates and treated with saikosaponin B2 for 48 hours. MTT (3-(4,5-dimethylthiozol-2-ly)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/mL) of 100 μL was added to each well. After incubation for 4 hours at 37°C, formazan crystals in viable cells were solubilized in 200 μL of DMSO. The soluble formazan product was spectrophotometrically quantified using an enzyme-linked immunosorbent assay reader at 570 nm.

2.7. Quantification of HCV RNA
Huh-7.5.1/pSGR-JFH1 stable cells were seeded into a 24-well plate at a density of 5 × 10⁴ cells per well and then incubated with growth medium containing 0.2% DMSO, with or without the indicated concentrations (10 or 100 μM) of saikosaponin B2 for 48 h. Then cells were lysed with the RNeasy Mini kit (Qiagen). The RNA concentration was determined using a NanoDrop spectrophotometer. For comparative quantification, we used TaqMan Fast virus one-step quantitative real-time PCR kit and the StepOnePlus Real-Time PCR system (Thermo Fisher Scientific Inc., Waltham, MA). Quantitative PCR was performed using HCV-specific primer 5′-CGGGAGAGCCATAGTGG-3′ (forward) and 5′-AGTACCACAAGGCCTTTCG-3′ (reverse).

2.8. Transfection and luciferase assay
For determination of HCV replication, stable Huh-7.5.1/pFK-I$_{389}$neo-ubi-luc-NS3-3′/5.1 cells were seeded into a 96-well plate at a density of 1 × 10⁴ cells per well and incubated with growth medium containing 0.2% DMSO, with or without the indicated concentrations (10 or 100 μM) of saikosaponin B2 for 48 hours. Firefly luciferase activity was determined according to the manufacturer's instructions (Promega, Madison, WI, USA).

For determination of HCV polyprotein translation, Huh-7.5.1 cells were seeded at a density of 1 × 10⁶ cells in a 10-cm dish and then transfected with 10 μg of RNA, which was in vitro transcribed from the pHCV-F.Luc-3′-UTR plasmid[28] in which the firefly luciferase gene was driven by the HCV internal ribosome entry site,[29] using the DMRIE-C transfection reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). After 24 hours, the transfected Huh-7.5.1 cells were trypsinized and plated into a 96-well plate at a density of 1 × 10⁴ cells per well and incubated with complete growth medium containing 0.2% DMSO with or without saikosaponin B2 for 48 hours. The cells were subjected to luciferase assay as described earlier.

2.9. HCV pseudotype particle production and infectivity inhibition assay
The luciferase-based HCV pseudotyped retroviral particles were produced through the transfection of plasmid DNA encoding HCV (genotypes 1b or 2a) glycoproteins with the Env-deficient HIV vector (pNL4-3-Luc-R-E-) carrying a luciferase reporter gene into the 293T producer cells. Supernatants containing the pseudotyped particles were collected 72 hours after transfection, filtered through a 0.45-μM pore-size filter (Millipore, Cork, Ireland), aliquoted, and frozen for later use. Huh-7.5.1 cells were inoculated with an equal number of viral particles, treated with 10 or 100 μM of saikosaponin B2 or DMSO control (0.2%) for 48 hours, and then subjected to luciferase assay.

2.10. Drug inhibition study
Stable Huh-7.5.1/pFK-I$_{389}$neo-ubi-luc-NS3-3′/5.1 cells were seeded into a 96-well plate at a density of 1 × 10⁴ cells per well and then incubated with indicated concentrations (10 or 100

μM) of saikosaponin B2 or DMSO control for 48 hours. Firefly luciferase activity was determined as described previously.

Serial 10-fold dilutions of the compound were subsequently prepared in culture medium. Huh-7.5.1 cells infected with J6/JFH(p7-Rluc2A) viral particles, either wild type or resistance-associated substitutions (RASs) (NS5A-L31M, -Y93H, or -L31M/93H), were incubated with serial 10-fold dilutions of the compound in 96-well plates and assayed for luciferase activity 48 hours later. The 50% effective concentration (EC_{50}) values were obtained through graphic interpolation of the concentration of the compound required for a 50% reduction in the luciferase activity in the linear portion of the curve.

Huh-7.5.1 cells were seeded in a 96-well plate at a density of 1×10^4 cells per well. After 24 hours, Huh-7.5.1 cells were pretreated for 1 hour with 50 μL of the fixed concentration of daclatasvir (10 pM), with or without increasing concentrations (1, 10, 100 μM) of saikosaponin B or an equivalent volume of DMSO, diluted in complete growth medium. The cells were then incubated with an equal volume of J6/JFH (p7-Rluc2A), wild type or RASs (NS5A-L31M, -Y93H, or -L31M/Y93H). The infection was allowed to proceed in the presence or absence of saikosaponin B2 and daclatasvir for 48 hours, followed by luciferase assay.

2.11. Statistical analysis

Data analysis was performed using the GraphPad Prism 5 (GraphPad Software, Inc. La Jolla, CA, USA). One-way analysis of variance was used to test for differences in means between groups, and a posthoc *t* test was used for comparisons. A $p < 0.05$ was considered statistically significant.

3. RESULTS

3.1. Inhibition of HCV infectivity by saikosaponin B2

Purified natural compounds were screened for the suppression of HCV production as described in Supplementary Fig. 1. Twenty nontoxic compounds showed strong HCV inhibition, among which four were available for further assays; three are flavonoids and were reported elsewhere,[32] and the fourth was saikosaponin B2, a glycoside (Fig. 1). To further elucidate the effect on HCV infection, Huh-7.5.1 cells were treated with saikosaponin B2 at 42 μg/mL followed by infection with J6/JFH(p7-Rluc2A) viral particles for 48 hours. Then, the cells were subjected to cell viability assay using MTT and luciferase assay representing viral infectivity. As shown in Fig. 2A, the compound did not affect cell viability at 42 μg/mL but suppressed more than 99% of HCV infectivity as represented by the percentage of luciferase activity relative to DMSO control (Fig. 2B). The dose that resulted in a 50% reduction of Huh-7.5.1 cells was 489.7 μM (Fig. 2C). Figure 3A shows the suppression of HCV infectivity by saikosaponin B2 in a dose-dependent manner, which attained a 54% reduction of infectivity at 10 μM. Figure 3B shows that saikosaponin B2 did not affect cell viability up to 100 μM.
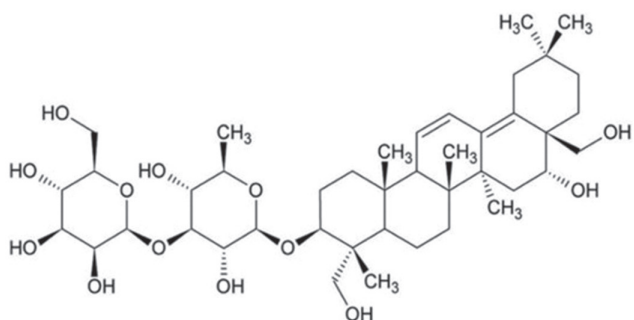
3.2. Inhibition of HCV polyprotein translation and replication by saikosaponin B2

To determine the effect of saikosaponin B2 on HCV polyprotein translation, we transfected Huh-7.5.1 cells with in vitro-transcribed pHCV-Fluc-3′-UTR RNA; the cells were then incubated in a medium containing 0.2% DMSO or the indicated concentrations of saikosaponin B2 for 48 hours. Luciferase was expressed, and its activity represented HCV polyprotein translation as a result of 3′-UTR as a strong stimulator of HCV polyprotein translation.[28] As shown in Fig. 4A, saikosaponin B2 suppressed HCV polyprotein translation at 100 μM, a dose that is still nontoxic to Huh-7.5.1 cells but results in 37% reduction in HCV translation. We next determined whether saikosaponin B2 affected HCV replication through the transfection of pFK-i389-Fluc-Ubi-Neo-NS3-3′.5.1 (Con1, genotype 1b) or
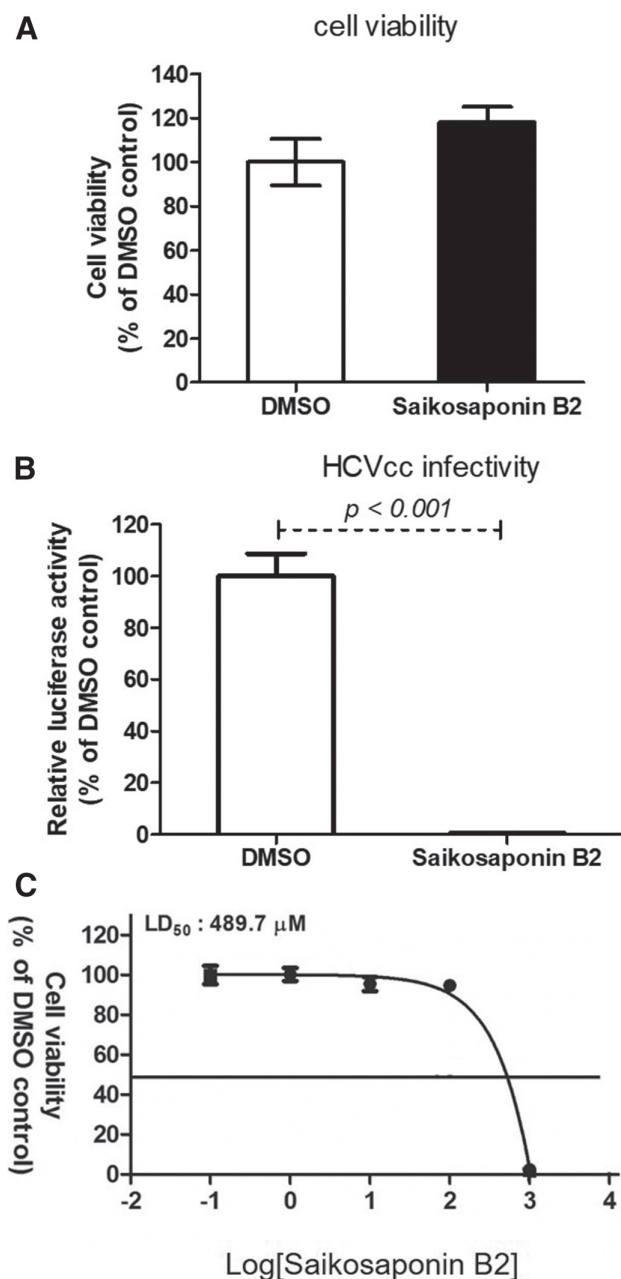

Fig. 2 Inhibitory effect of saikosaponin B2 on hepatitis C virus (HCV) infectivity. Huh-7.5.1 cells were treated with DMSO or saikosaponin B2 at 42 μg/mL for 1 h followed by infection with J6/JFH(p7-Rluc2A) virus for 48 h. The cells were subjected to MTT assay for cell viability (A), luciferase assay representing viral infectivity (B), and lethal dose 50 (LD_{50}) that resulted in 50% inhibition of cell growth.

Fig. 1 Structure of saikosaponin B2. Saikosaponin B2, a glycoside bearing a triterpene structure, isolated from *Bupleurum kaoi* roots (*B. chinensis*, Apiaceae), a pla used in East Asia for the treatment of liver disease.
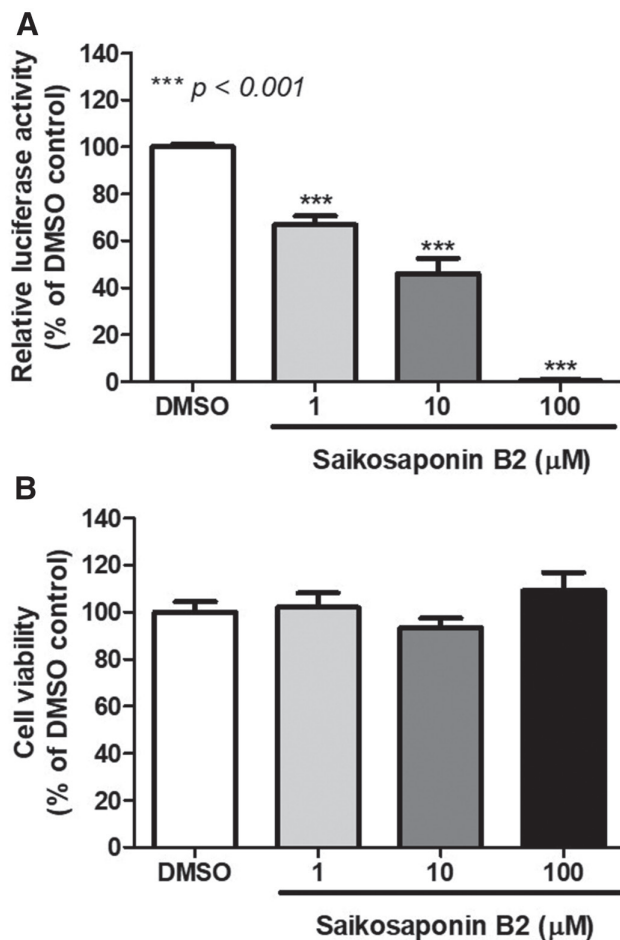
Fig. 3 Dose-dependent inhibition of saikosaponin B2 on hepatitis C virus (HCV) infectivity. As described in Fig. 2, Huh-7.5.1 cells were treated with DMSO or the indicated concentrations of saikosaponin B2 (1, 10, and 100 μM) for 1 h, followed by infection with J6/JFH(p7-Rluc2A) virus for 48 h. Then, the cells were subjected to (A) Renilla luciferase assay and (B) MTT assay to determine the HCV infectivity and cell viability, respectively. ***$p < 0.001$ vs control, by Student's *t* test.

pSGR-JFH1 (genotype 2a) plasmid into Huh-7.5.1 cells to establish stable cells, in which the former viral replication was represented by luciferase activity and the latter was represented by HCV RNA level. As shown in Fig. 4B, C (genotype 1b and genotype 2a), saikosaponin B2 suppressed HCV RNA replication in a dose-dependent manner. For genotype 1b (Con1), 10 μM resulted in 34% reduction in replication, and 100 μM resulted in 55% reduction; for genotype 2a (JFH1), 10 μM resulted in 17% reduction, and 100 μM resulted in 71% reduction.

3.3. Inhibition of HCV entry into Huh-7.5.1 cells by saikosaponin B2

Pseudotype HCV was produced through cotransfection of a DNA vector encoding HCV (genotypes 1b or 2a) glycoprotein with the Env-deficient HIV vector (pNL4-3-Luc-R-E-) carrying a luciferase reporter gene into the 293T producer cells. Huh-7.5.1 cells were inoculated with Con1 or JFH1 pseudotypes in the presence or absence of saikosaponin B2. As shown in Fig. 5A, B (Con1 and JFH1), entry of pseudotyped HCV was inhibited by saikosaponin B2, with 78% reduction at 10 μM for Con1 and 69% reduction at 100 μM for JFH.

3.4. Saikosaponin B2 circumvents daclatasvir-induced RASs

The problem of treating HCV with DAAs led to the emergence of RASs.[30] We tested the effect of saikosaponin B2 on RASs of NS5A-Y93H, L31M, and Y93H/L31M, which are known to be induced by failed HCV treatment with daclatasvir.[33] Huh7.5.1
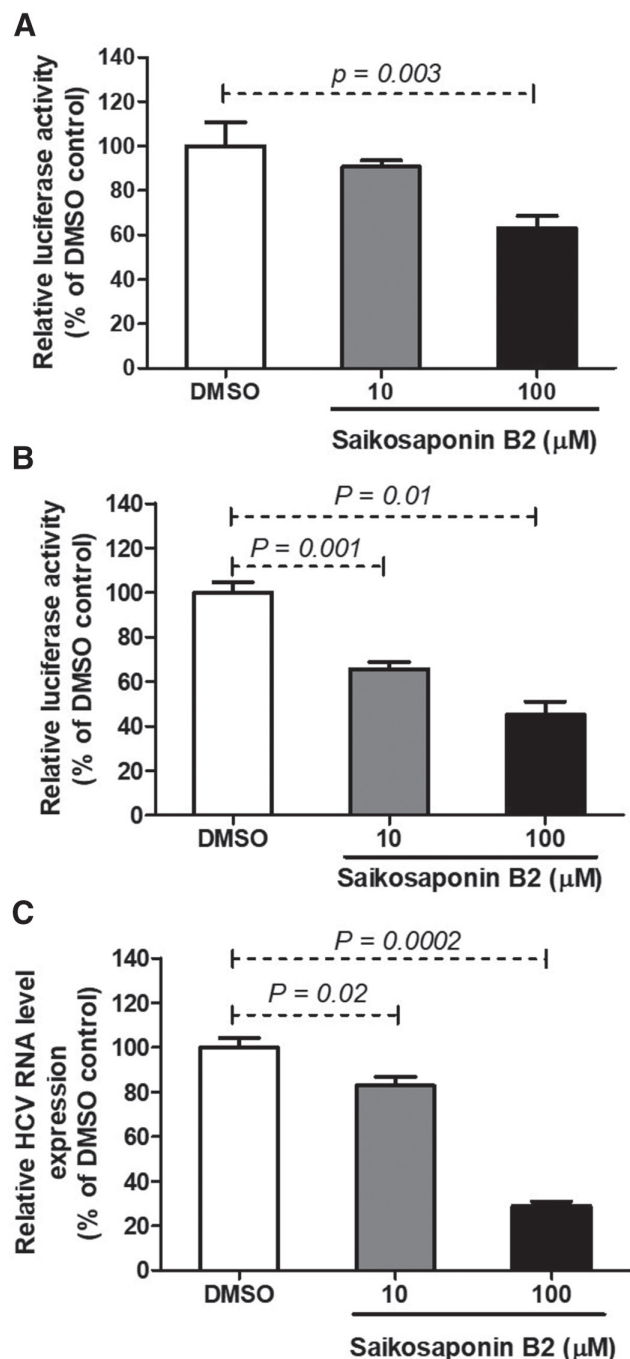

Fig. 4 Effect of saikosaponin B2 on hepatitis C virus (HCV) translation and replication. A, Huh-7.5.1 cells were transfected with in vitro-transcribed pHCV-Fluc-3′-UTR RNA and then incubated with medium containing 0.2% DMSO or the indicated concentrations of saikosaponin B2 for 48 h. Translation efficiency was estimated by the luciferase activity and expressed as a percentage of the DMSO control. B, Huh-7.5.1/pFK-I$_{389}$ luc-ubi-neo/NS3-3/5.1 stable cells were incubated with medium containing 0.2% DMSO or the indicated concentrations of saikosaponin B2 for 48 h. HCV replication efficiency was estimated by the luciferase activity and expressed as percentage of the DMSO control. C, Huh-7.5.1/pSGR-JFH1 stable cells were incubated with medium containing 0.2% DMSO or the indicated concentrations of saikosaponin B2 for 48 h. HCV replication efficiency was determined through quantitative real-time PCR and expressed as percentage of the DMSO control. Columns and bars represent means and SD, respectively, of at least three independent transfections. All *p* values were determined by paired *t* test.

cells were infected with wild-type or RAS-bearing J6/JFH(p7-Rluc2A) (wt, L31M, Y93H, or L31M/Y93H) and then treated with saikosaponin B2. Saikosaponin B2 inhibited all three RASs (wt > Y93H > L31M > L31M/Y93H), as indicated by EC$_{50}$
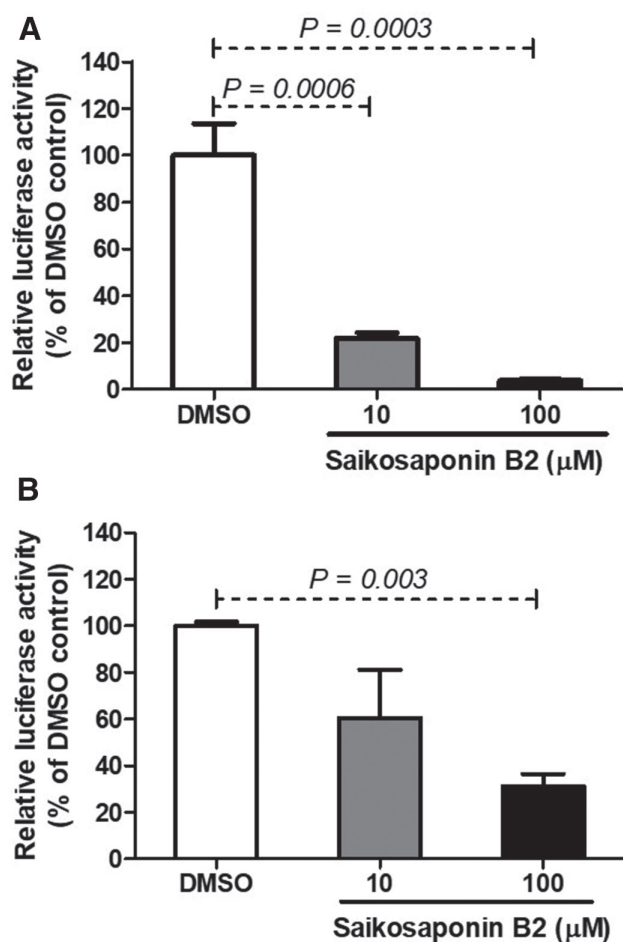
Fig. 5 Effect of saikosaponin B2 on hepatitis C virus (HCV) entry into cells. A, Huh-7.5.1 cells were inoculated with (A) Con1 or (B) JFH1 pseudotypes in the presence of the indicated concentrations of saikosaponin B2 or an equivalent volume of DMSO. Infection efficiency was determined by luciferase activity and expressed as percentage of the DMSO control. Data shown were the mean and error values of a minimum of two independent experiments in triplicate. The *p* value was determined by paired *t* test.

(Fig. 6A). The compound also had a circumventing effect on the daclatasvir-resistant mutant L31M, with a significant reduction of viral replication at the low dose of 1 μM in combination with 10 pM of daclatasvir (30% reduction for L31M vs 60% reduction for wt), and it showed a varied circumventing effect on daclatasvir-resistant mutants at 10 μM in combination with 10 pM of daclatasvir (L31M > Y93M > L31M/Y93M) (Fig. 6B). The combined treatments reached the optimal effect at 100 μM against wt or RASs (Fig. 6B). These results suggested that saikosaponin B2 may combine with DAAs to treat resistant strains of HCV infection.

4. DISCUSSION

Persistent HCV infection is a major global health issue, leading to cirrhosis and hepatocellular carcinoma, which is the most common indication for liver transplantation in western countries.[5,34] The disease progresses treacherously, and the previous treatment of pegylated interferon and ribavirin is unsatisfactory and carries considerable side effects. There is no vaccine against HCV. Only recently, with advances in drugs targeting the HCV life cycle, DAAs have been developed and greatly improved the therapeutic effect. However, DAAs remain expensive and will probably not be available for all patients worldwide. For this reason, efforts to develop new and inexpensive anti-HCV molecules continue.

Phytochemicals as antiviral agents have become critical in recent years.[35] Flavonoids or flavanoids such as the catechins are the most common polyphenolic compounds in the human diet with anti-HCV activity and are found ubiquitously in plants.[11] In this study, we screened compounds from National Institute of Chinese Medicine Library in Taiwan and found saikosaponin B2 to exhibit inhibitory effects on HCV replication, polyprotein translation, and entry into cells (Figs. 4 and 5). The compound also had inhibitory effects on HCV NS5A RAS replication, alone or in combination with daclatasvir.

Saikosaponins have been shown to inhibit a variety of viral infections. Saikosaponin A inhibits influenza A replication.[36] Saikosaponin D inactivates measles virus and herpes simplex virus.[37] Lin et al[19] reported that saikosaponin B2 inhibits HCV entry by preventing HCV E2 attachment to the cell membrane but does not affect viral replication or translation. However, we discovered that it inhibited both HCV replication (Con1 at 10 μM and JFH1 at 100 μM) and translation (at 100 μM) (Fig. 4); the difference probably resulted from the different HCV Jc1 strain and drug dose used in their study. The Jc1 genome is derived from JFH1. As shown in Fig. 4, saikosaponin B2 inhibited Con1 replication at 10 μM (Fig. 4B) but JFH1 at 100 μM (Fig. 4C). The drug dose was elevated to 100 μM to inhibit JFH1 RNA translation (Fig. 4A). Lin et al also demonstrated that different saikosaponins had different EC_{50} values for the Jc1 strain of HCV: saikosaponin A is 1.4 μM, saikosaponin B2 is 16.13 μM, saikosaponin C is 86.12 μM, and saikosaponin D is 2.8 μM. It is worth noting that the EC_{50} of saikosaponin B2 for J6/JFH1 is 8.93 μM and of saikosaponin B2 for Jc1 is 16.13 μM. These results suggest versatile actions of various saikosaponins against HCV with different isolates. Furthermore, similar to Lin et al, we also demonstrated that saikosaponin B2 inhibited HCV entry (Fig. 5); however, we found that that this compound also inhibited HCV RNA replication in Con1 strain (Fig. 4).

A number of purified plant compounds have exhibited to anti-HCV activities in the recent decade, but their mechanisms of action remain unclear. As with saikosaponin B2, inhibition of HCV replication was the main mechanism of action; for example, when corilagin,[38] 3-hydroxy caurilignan C,[39] and quercetin[40] act on NS3 protease to inhibit HCV replication. Ladanein[12] and EGCG[41] are potent entry inhibitors, and quercetin[14] might be more active on the assembly than on the replication step. Because the HCV RNA 3′ untranslated region strongly stimulates polyprotein translation directed by the internal ribosomal entry site,[28] we used in vitro-transcribed RNA from the pHCV-F.Luc-3′-UTR plasmid to transfect Huh-7.5.1 cells and found that saikosaponin B2 interfered with HCV polyprotein translation (Fig. 5A), suggesting that it may interact with HCV RNA and prevent RNA from entering the endoplasmic reticulum for polyprotein synthesis. This is the first study showing that natural compounds inhibit HCV polyprotein translation by suppressing 3′-UTR.

HCV treatment has advanced considerably with the development of DAAs; however, resistant strains remain a problem. NS5A Y93H and L31M mutants are selected during HCV treatment with daclatasvir.[33] We tested saikosaponin B2 and found that it had satisfactory EC_{50} for mutant strains of J6/JFH1 HCV (NS5A-L31M: 15.54 μM, NS5A-Y93H: 12.51 μM) in comparison with NS5A-wt (8.93 μM). EC_{50} of saikosaponin B2 for the Jc1 strain is 16.13 μM.[19] HCV-NS5A-wt and -L31M (or -Y93H) possess the same genetic background except the 31st (or 93rd) amino acid of NS5A. It appears that one of the actions of saikosaponin B2 is to inhibit NS5A but in a pharmacological mechanism distinct from that of daclatasvir. Thus, saikosaponin B2 may be a potential NS5A inhibitor for patients with HCV from mutant strains, which are difficult to treat.

In conclusion, saikosaponin B2 inhibits HCV entry, polyprotein translation, and RNA replication. Further translational study is required to prove the effect of saikosaponin B2 and design new combination treatments for patients with HCV.
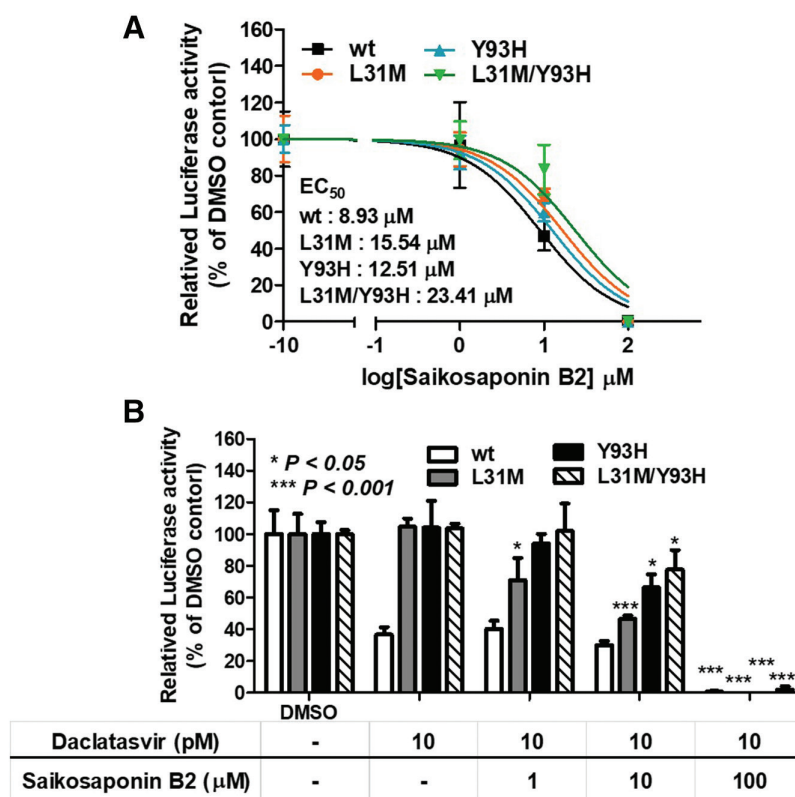
Fig. 6 Replication inhibition of saikosaponin B2 in combination with daclatasvir on hepatitis C virus (HCV) with NS5A RASs. A, Huh-7.5.1 cells were infected with wild-type or the indicated RAS-bearing J6/JFH(p7-Rluc2A) viral particles and then treated with serial 10-fold dilutions of the indicated saikosaponin B2 to determine the antiviral effects. The EC_{50} values were obtained through graphic interpolation of the compound concentration resulting in 50% HCV infection inhibition in the linear portion of the curve. B, Huh-7.5.1 cells infected with wild-type or the indicated RAS-bearing J6/JFH(p7-Rluc2A) viral particles were treated with a fixed concentration of daclatasvir (10 pM) and increasing concentrations (1, 10, 100 μM) of saikosaponin B2 to determine the antiviral effect of combination treatment. *$p < 0.05$, ***$p < 0.001$ vs daclatasvir (10 pM) only of corresponding group, by Student's *t* test.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jcma.2018.01.013.

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