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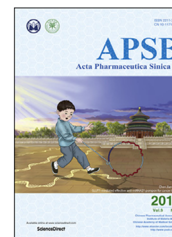
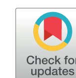


ORIGINAL ARTICLE

Up-regulation of glycolipid transfer protein by bicyclol causes spontaneous restriction of hepatitis C virus replication



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KEY WORDS

Bicyclol;
Hepatitis C virus;
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Protein interaction

Abstract Bicyclol is a synthetic drug for hepatoprotection in clinic since 2004. Preliminary clinical observations suggest that bicyclol might be active against hepatitis C virus (HCV) with unknown mechanism. Here, we showed that bicyclol significantly inhibited HCV replication *in vitro* and in hepatitis C patients. Using bicyclol as a probe, we identified glycolipid transfer protein (GLTP) to be a novel restrictive factor for HCV replication. The GLTP preferentially bound host vesicle-associated membrane protein-associated protein-A (VAP-A) in competition with the HCV NS5A, causing an interruption of the complex formation between VAP-A and HCV NS5A. As the formation of VAP-A/NS5A complex is essential for viral RNA replication, up-regulation of GLTP by bicyclol reduced the level of VAP-A/NS5A complex and thus inhibited HCV replication. Bicyclol also exhibited an inhibition on HCV variants resistant to direct-acting antiviral agents (DAAs) with an efficacy identical to that on wild type HCV. In combination with bicyclol, DAAs inhibited HCV replication in a synergistic fashion. GLTP appears to be a newly discovered host restrictive factor for HCV replication, Up-regulation of GLTP causes spontaneous restriction of HCV replication.

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1. Introduction

Bicyclol (4,4′-dimethoxy-5,6,5′,6′-bis(methylenedioxy)-2-hydroxymethyl- 2′-methoxycarbonyl biphenyl) is a synthetic drug originated from *Schisandra chinensis*, an herb used to treat hepatitis in traditional Chinese medicine (TCM) in general[1,2]. It was approved as a hepatoprotectant by the Chinese Food and Drug Administration (FDA) for treatment of liver injury in 2004[1]. Oral administration of bicyclol significantly ameliorates liver damage in patients with chronic hepatitis, causing reduction of liver alanine aminotranferease and aspartate aminotransferase. It is also effective in patients with acute liver damage induced by alcohol, drugs, or ischemia/reperfusion injury[1,3–5]. The mechanism of bicyclol in its action against liver injury links to its anti-inflammatory and anti-oxidative effects[1]. Severe side-effect of bicyclol was not found and no individuals discontinued bicyclol treatment due to adverse events during 6-month treatment course, suggesting that bicyclol is a safe drug[1–3,5]. Interestingly, some clinical observations showed that bicyclol might be an antiviral agent for hepatitis C[1,5]. In a phase IV clinical trial in China, chronic hepatitis C (CHC) patients who have failed to the standard therapy (interferon-α plus ribavirin) were enrolled to receive bicyclol treatment. After 6-month treatment with bicyclol, both HCV RNA and liver transaminases levels decreased in the patients[1,6]. However, the mechanism remains unclear.

After seeing the anti-HCV activity of bicyclol *in vitro* and in hepatitis C patients, we used bicyclol as a probe in an attempt to explore the antiviral molecular mechanism of bicyclol. What presented below shows that glycolipid transfer protein (GLTP) is a novel HCV restrictive factor in hepatocytes, and up-regulated expression of GLTP by bicyclol causes spontaneous clearance of HCV. We consider the study shed new light on our understanding of TCM in host action against viral invasion.

2. Materials and methods

2.1. Cells and virus

Huh7.5 cells and the plasmid pFL-J6/JFH/JC1 containing the full-length chimeric HCV complementary DNA (cDNA) were kindly provided by the Vertex Pharmaceuticals Inc. (Boston, MA, USA). The drug-resistance viruses with site-directed mutation were derived from plasmid pFL-J6/JFH/JC1. HCV virus stock was prepared as described previously[7]. Huh7.5 cells, 293T/17 cells (from ATCC) and GS4.3 replicon cells were cultured as described before[7]. Primary human hepatocytes (PHHs) were from the ScienCell Research Laboratories (San Diego, CA, USA) and cultured according to the manufacturer's instructions.

2.2. Agent

Bicyclol was from the Beijing Union Pharmaceutical Company (Beijing, China) with purity over 99%. Sofosbuvir (HY-15005S), simeprevir (HY-10241) and telaprevir (VX-950, HY-10235) were from the MedChemExpress (Princeton, NJ). Interferon-α-2b (Intron A, IFN) was from the Schering Plough (Brinny) Company (NJ, USA). Antibodies for GLTP (sc-242912 and sc-242913) and for VAP-A (sc-48698), shRNA plasmid for GLTP (sc-95836-SH), shRNA plasmid-A (plasmid control, sc-108060) and secondary antibody (sc-2354) were from the Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for HCV core (ab2740) and NS3 (ab13830) were from the Abcam (Cambridge, UK). Antibodies for beta-Actin (3700s) and His-tag (2365s) and secondary antibodies (7074S and 7076S) were from the Cell Signaling Technology (Danvers, MA, USA). Antibody for HCV NS5A (pab24633) was from the Abnova (Taipei, Taiwan).

2.3. Anti-HCV activity of bicyclol in Huh7.5 cells and primary human hepatocytes

Huh7.5 cells infected with wild type (WT) or mutant HCV (45 IU/cell) were simultaneously treated with bicyclol or approved drugs. Seventy-two hours later, intracellular RNAs were extracted with RNeasy Mini Kit (74106, Qiagen) and quantified with qRT-PCR, and intracellular proteins were extracted using the CytoBuster Protein Extraction Reagent (PER, Novagen, Madison, WI, USA) with 1 mmol/L protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and analyzed with western blot (WB). Cell survival of the Huh7.5 cells were evaluated at 72 h using a tetrazolium assay (MTT, Amresco)[7].

Primary human hepatocytes inoculated in a poly-L-lysine coated 12-well plate were infected with HCV viral stock (2000 IU/cell), and were simultaneously treated with 10 or 50 μmol/L bicyclol or 0.1 μmol/L VX-950. After 7 days infection, intracellular RNAs were extracted with RNeasy Mini Kit and quantified with qRT-PCR, and intracellular proteins were extracted with PER and analyzed with WB.

2.4. Anti-HCV activity of bicyclol in HCV-positive Huh 7.5 cells and GS4.3 replicon cells

HCV-positive Huh7.5 cells after infection with HCV for over 10 days, or GS4.3 replicon cells were treated with bicyclol. Cultural supernatants were replaced with fresh culture media containing bicyclol every other day. On days 2, 6, 10 and 14, intracellular RNAs were extracted with RNeasy Mini Kit and quantified with qRT-PCR, and intracellular proteins were extracted with PER and analyzed with WB. A protease inhibitor teleprevir (VX-950, 0.1 μmol/L) served as a positive control.

2.5. Drug combination treatment in Huh7.5 cells

Huh7.5 cells were infected with HCV (45 IU/cell) and treated with bicyclol or approved drug alone, or in combination with two drugs at the ratio of 1:1. After incubation for 72 h, intracellular RNAs and proteins were extracted and evaluated with qRT-PCR and WB, respectively. The combination index (CI) was calculated by the Chou-Talalay method using CompuSyn version 1.0, in which CI $>$ 1 indicates antagonism, CI $=$ 1 indicates addition, while

CI < 1 suggests synergy between the two drugs[8,9]. Another evaluation indication is a dose-reduction index (DRI), in which DRI > 1 indicates a favorable reduction in toxicity when maintaining the therapeutic efficacy, whereas DRI < 1 indicates no reduction of toxicity in combination[9,10].

2.6. *Animal experiment*

Fifty KM mice (22.0 g ± 2.0 g) were from the Beijing HuaFu-Kang Biological Technology Co. Ltd. (Beijing, China). Mice were randomly divided into five groups with five male and five female mice in each group, and administered intragastrically with bicyclol (25, 75 and 250 mg/kg) or VX-950 (375 mg/kg) dissolved in 0.5% carboxymethyl cellulose sodium (CMC-Na) or solvent control twice a day for 7 days. The body weights were measured at days 0, 4, and 7. The mice were sacrificed and the liver tissues were collected at 4 h post the last administer. Liver total RNAs were extracted with TRIzol Reagent (Invitrogen) and quantified with qRT-PCR, liver total proteins were extracted with TPER (Thermo Scientific) and analyzed with WB. Animal experiments were conducted following the National Guidelines for Housing and Care of Laboratory Animals and performed in accordance with protocol approved by the Institutional Animal Care and Use Committee.

2.7. *Clinical trial of bicyclol in CHC patients*

Seventeen patients diagnosed with CHC (HCV-1b) had been enrolled in this study between May 2016 and Sep 2017 in the Nanjing Second Hospital in Nanjing, China (Registration number: ChiCTR-OPh-17011456; and registration website: www.chictr.org.cn). All of the patients were diagnosed as HCV-1b infection, with HCV RNA load above 2000 IU/mL and serum HCV antibody positive. CHC patients with co-infection of HBV or HIV-1, or with liver cirrhosis or hepatoma, or with non-genotype 1 HCV infection were not included. Those who had received anti-HCV therapy (for instance, ribavirin/interferon regimen or direct-acting antiviral agents (DAAs) treatment) within 3 months before the study were excluded as well. The average age of the 17 patients was 50.1 ± 12.3 (between 26 and 68). Of the 17 patients, 4 were men (aged 48.5 ± 16.5 years) and 13 were women (aged 50.5 ± 11.4 years). The patients orally received bicyclol treatment (50 mg, tid; Beijing Union Pharmaceutical Company, Beijing, China) for 3 months. The therapeutic efficacy of bicyclol was evaluated by measuring HCV RNA viral load, liver enzymes, kidney function, blood cells, as well as hemoglobin. The primary end point of the study was the anti-HCV efficacy and adverse effects of bicyclol. All the patients signed the informed consent. The clinical trial study was approved by the Ethics & Research Committee of the Nanjing Second Hospital (2016-LS-ky002).

2.8. *Plasmids construction and mutagenesis*

The open reading frame encoding human *GLTP* (NCBI reference sequence: NM_016433.3) was sub-cloned and inserted into an expression vector pcDNA3.1(+) with cloning sites *Hin*d III/*Bam*HI. The plasmid GLTP-His or GLTP-HA expressed a full-length form of human GLTP with His-tag or HA-tag at the C-terminus was constructed by inserting the study sequences into a pcDNA3.1(+) vector. GLTPs with site-directed mutation were obtained with a Fast Site-Directed Mutagenesis Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The primers used in the experiments were shown in Supporting Information Table S1.

2.9. *Construction of reporter vector*

The oligonucleotide pairs of wild type or mutant type *GLTP* 3′-UTR for miR-449b targeted or mismatched sequences (Supporting information Table S2) were synthesized by the Sangon Biotech (Shanghai) Co., Ltd. (China) and were then cloned into the pmirGLO dual-luciferase miRNA target expression vector (Promega) with the PmeI and XbaI restriction site according to manufacturer's instructions.

2.10. *The effect of miR-449b on the endogenous GLTP expression*

Huh7.5 cells were transfected with 50 nmol/L of miR-449b mimic, or with 100 nmol/L of miR-449b inhibitor (RiboBio) using Lipofectamine RNAiMAX (Invitrogen). 50 nmol/L of mimic negative control or 100 nmol/L of inhibitor negative control (RiboBio) was as a control. Intracellular RNA and proteins were detected in 48 h with qRT-PCR and WB, respectively.

2.11. *Immunoprecipitation assay*

After being treated, the Huh7.5 cells were collected and lysed in PER. The cell lysates of HCV-positive Huh7.5 cells and lysates of naïve Huh7.5 cells transfected with expression vector (or plasmid control) were mixed at 1:1 ratio. The mixtures were incubated with 4 μg of the GLTP antibody (sc-242913) or VAP-A antibody (sc-48698) for 16 h at 4 °C, followed by addition of 50 μL protein G agarose (Roche Applied Science) and continuous incubation for 3 h. Then, the immunoprecipitates were washed 4 times with cold DPBS in brief centrifugation. The pellets were resuspended with 50 μL 2 × loading buffer, and were boiled for 5 min. After brief centrifugation, the supernatants were collected and the proteins were analyzed with WB as previously described[7].

2.12. *Luciferase reporter assays*

293T/17 cells in a 96-well plate were co-transfected with 100 ng of recombinant pmirGLO plasmid containing wild type (WT) or mutant (Mut) *GLTP* 3′-UTR sequences and 50 nmol/L of miR-449b mimic (RiboBio) using Lipofectamine 2000 (Invitrogen). The cells co-transfected with 100 ng of recombinant pmirGLO plasmid and 50 nmol/L of mimic negative control (RiboBio) severed as a control. The fluorescent intensity of firefly luciferase and *Renilla* luciferase were detected stepwise by the Enspire Multimode Reader (PerkinElmer) using the Dual-Glo luciferase assay system (Promega) in 24 h.

2.13. *The quantitation of mRNA*

The total RNA extracted from cells was analyzed using the AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster, CA, USA). Fluorescent signals were detected with 7500 fast real-time PCR system (Applied Biosystems, Foster, CA, USA) according to the manufacturer's procedure. All quantifications were normalized to the level of the internal control gene, glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), the levels of HCV RNA were analyzed with the $2^{-\Delta\Delta CT}$ method, and a value

of half maximal effective concentration (EC_{50}) was calculated with the Reed-Muench Method. For qualification of mRNA with SYBGreen method, the primers showed in Supporting information Table S3. The mRNA was qualified using a Super-Script III Platinum SYBR Green One-Step qRT-PCR Kit according to the manufacturer's procedure.

2.14. *The quantitation of miRNA*

Intracellular miRNAs extracted from cells with TRIzol Reagent (Invitrogen) were reverse-transcripted into cDNA using Taqman miRNA RT kit (Applied Biosystems). The cDNA was then detected using Taqman Universal MMIX II (Applied Biosystems) with Taqman probes for hsa-miR-449b (001608, Applied Biosystems) and internal control U6 snRNA (001973, Applied Biosystems).

2.15. *Western blot*

The proteins were detected with Western blot (WB). Briefly, after SDS-PAGE and trans-membrane, the target proteins were accordingly probed with first antibody for HCV Core, HCV NS3, GLTP, His-tag, HA-tag, VAP-A, or NS5A, respectively. Anti-actin antibody was used as an internal control. The proteins were detected using the Immobilon Western Chemiluminescent HRP Substrate (Millipore, Inc.) with ChemiDo XRS gel imager system (Bio-Rad, CA). Protein signal intensity was scanned with the Gelpro32 software, and a ratio of interested protein to internal control protein Actin was calculated and normalized as 1.00 for the control group.

2.16. *Quantification and statistical analyses*

Data shown in the histogram were the mean $\pm$ standard deviation of over 3 independent experiments. Data were analyzed using the ANOVA analysis followed by the Student's *t*-test. The level of significance was set at $P < 0.05$. Statistical analysis for clinical results was done with SPSS 15.0 software.

3. Result

3.1. *Bicyclol inhibits HCV replication in vitro*

The anti-HCV effect of bicyclol was first analyzed *in vitro*. Huh7.5 cells were infected with HCV and were simultaneously treated with bicyclol for 72 h, Bicyclol decreased HCV RNA in a dose-dependent manner (Fig. 1A, left), with the inhibitory effect being statistically significant ($P < 0.01$) when bicyclol was at 10 μmol/L. The result was validated at protein level by measuring either HCV Core or NS3 protein (Fig. 1A, right).

To learn whether bicyclol works for the established HCV infection, we tested the anti-HCV activity of bicyclol in the HCV-positive Huh7.5 cells which were infected with HCV for over 10 days. The cells were treated with bicyclol, and during the cultivation the supernatants were replaced with fresh culture media containing bicyclol every other day. The intracellular HCV RNA and proteins were detected on days 2, 6, 10, and 14 post-treatment. The results showed that bicyclol significantly reduced HCV RNA (Fig. 1B, left) and proteins (Fig. 1B, right) in the HCV-positive Huh7.5 cells in a dose-dependent manner, and the reduction was detected even when bicyclol was at 2 μmol/L (Fig. 1B). Bicyclol at 50 μmol/L exhibited an inhibitory effect on HCV replication equal to that of VX-950 at 0.1 μmol/L (Fig. 1B). The results were supportive to the findings of bicyclol in clinic, showing an anti-HCV effect of bicyclol in mono-therapy in hepatitis C patients[1].

To further validate the anti-HCV activity of bicyclol *in vitro*, the GS4.3 cells, a human hepatoma Huh-7 cell line carrying an HCV sub-genomic replicon I 377-3′del.S[11], were used. The sub-genomic replicon has an in-completed HCV propagation cycle and is in lack of the early as well as late replication events. The results showed that bicyclol also inhibited HCV replication at RNA (Fig. 1C, left) and protein (Fig. 1C, right) levels in the GS4.3 cells, suggesting that bicyclol might inhibit HCV propagation in the middle stage of viral replication.

3.2. *Bicyclol inhibits HCV replication in CHC patients*

As bicyclol has been approved by the Chinese FDA for patients with viral hepatitis since 2004, a clinical trial was conducted in the Nanjing Second Hospital. In this study, 17 patients infected with HCV-1b were enrolled in the clinical trial. The general information for the patients is described in the Materials and Methods. The patients were treated with bicyclol at a dose of 50 mg (orally, tid, for 3 months), in an attempt to learn the therapeutic effect of bicyclol on HCV-1b infection as well as its adverse effects. The therapeutic efficacy was assessed by determining the blood HCV RNA viral load and liver enzymes. Among the 17 patients, one individual withdrew from the beginning of the study after signing the informed consent, one was lack of HCV RNA baseline information, and one was loss to follow up at week 12. Thus, the results presented were from 15 individuals. The blood HCV load measurements showed that treating the patients with bicyclol reduced blood HCV RNA load by 1.3 log (95.95%) after 3 months of bicyclol therapy, and the inhibition rate was in a time-dependent manner (Fig. 2A). HCV inhibition by bicyclol for each individual is shown as well (Supporting Information Fig. S1). Liver enzymes (AST and ALT) declined significantly after bicyclol treatment (Fig. 2B, $P < 0.01$, baseline *vs*. 3-month therapy). Significant changes in the blood cell counts, hemoglobin level and kidney function in response to bicyclol treatment were not observed (Fig. 2C).

3.3. *Bicyclol up-regulates the cellular GLTP through down-regulating microRNA-449b expression*

Then, the activity of bicyclol on HCV replicative enzymes was tested in the cell-free systems. Bicyclol showed no direct inhibition against HCV protease or helicase (data not show), suggesting that the HCV enzymes might not be the antiviral target of bicyclol. Thus, bicyclol's effect in HCV-infected cells was analyzed at gene and microRNA levels using microarray (Supporting Information Fig. S2). HCV infection increased expression of microRNA-449b (miR-449b) in Huh7.5 cells (Fig. S2A, left), but the level of *GLTP* mRNA went down (Fig. S2A, right). The negative correlation between the two factors caught our attention. Based on the computational calculation using the softwares TargetScan and miRanda the miR-449b was expected to directly bind the *GLTP* 3′-UTR site (Fig. S2B, up). We then validated the results with qRT-PCR and WB. The results showed that when the level of miR-449b increased in the HCV-infected Huh7.5 cells (Fig. 3A, left), the levels of *GLTP* mRNA (Fig. 3A, left) and protein (Fig. 3A, right) decreased (although the decrease of mRNA was moderate);

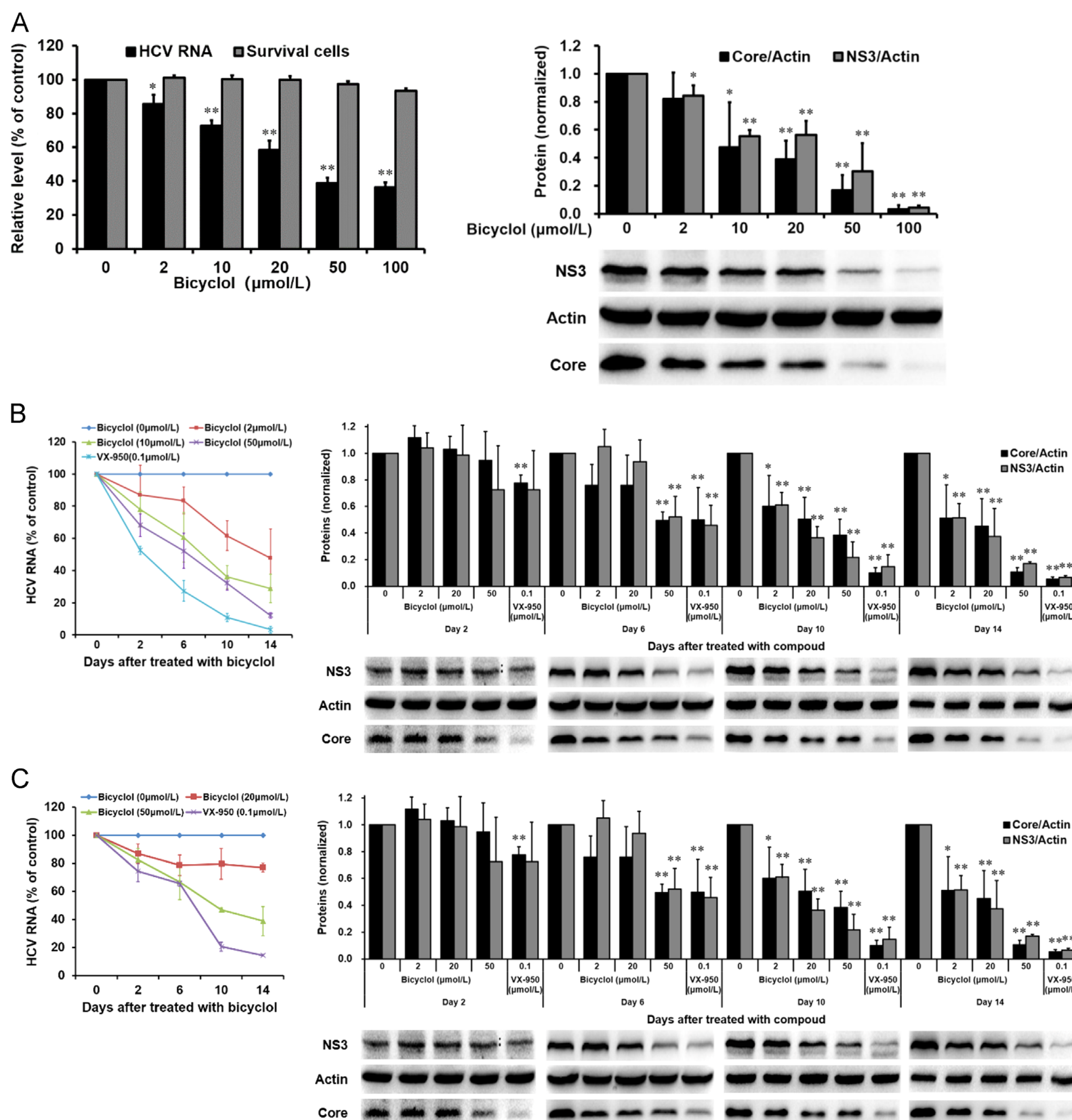

Figure 1 Bicyclol inhibits HCV replication *in vitro*. (A) Bicyclol inhibited HCV replication in the HCV-infected Huh7.5 cells. Huh7.5 cells were infected with HCV (45 IU/cell) and simultaneously treated with bicyclol. After 72 h incubation, intracellular RNA (left) and proteins (right) were detected with qRT-PCR and WB, respectively, and survival cells were measured with an MTT assay (left) ($n = 3$, *$P < 0.05$, and **$P < 0.01$, *vs.* solvent control). Bicyclol inhibited HCV replication in the HCV-positive Huh7.5 cells (B) or GS4.3 cells (C). HCV-positive Huh7.5 cells or GS4.3 cells were treated with bicyclol, and the culture supernatants were replaced every other day with fresh culture media containing bicyclol. On days 2, 6, 10, and 14, intracellular HCV RNA (left) and proteins (right) were detected. The VX-950 served as a positive control in the test ($n = 3$, *$P < 0.05$, or **$P < 0.01$ *vs.* solvent control). The protein bands showed the results of a representative experiment. The data presented are the mean ± standard deviation.

paralleled with which was an elevation of HCV RNA (Fig. 3A, left) and proteins (Fig. 3A, right, HCV Core and NS3). Treating the HCV-infected cells with bicyclol decreased the level of miR-449b (Fig. 3B, left, and Fig. S2C, left) and increased *GLTP* mRNA (Fig. 3B, left, and Fig. S2C, right) and protein (Fig. 2B, right) expression in the HCV-infected Huh7.5 cells.

To identify whether the expression of GLTP relates to miR-449b, a luciferase reporter assay was performed. The consensus binding sites of miR-449b in *GLTP* 3′-UTR (WT) or its mutant (Mut) (Fig. S2B, bottom) was cloned into the pmirGLO reporter vector. Results showed that miR-449b significantly reduced the relative luciferase/renilla intensity of the vector with the wild type

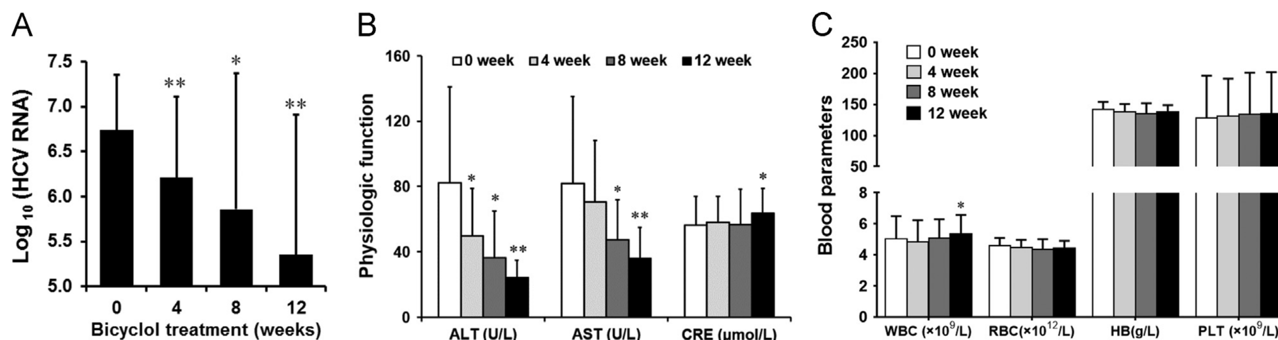

Figure 2 Bicyclol inhibits HCV replication in CHC patients at a dose of 50 mg (orally, tid, for 3 months, $n = 15$). (A) HCV RNA load (IU/mL) in blood, (B) Liver and kidney physiologic function, (C) Whole blood parameters. (*$P < 0.05$ and **$P < 0.01$ *vs*. 0 week). The data presented are the mean ± standard deviation. A paired Student's *t*-test was used. ALT, alanine aminotransferase (5.0–40.0 U/L); AST, aspartate aminotransferase (5.0–37.0 U/L); CRE, creatinine (44.0–106.0 μmol/L); WBC, while blood cell(4.0–10.0 × 10^9/L); RBC, red blood cell (3.50–5.50 × 10^{12}/L); HB, hemoglobin (110.0–160.0 g/L); PLT, platelet (100–300 × 10^9/L).

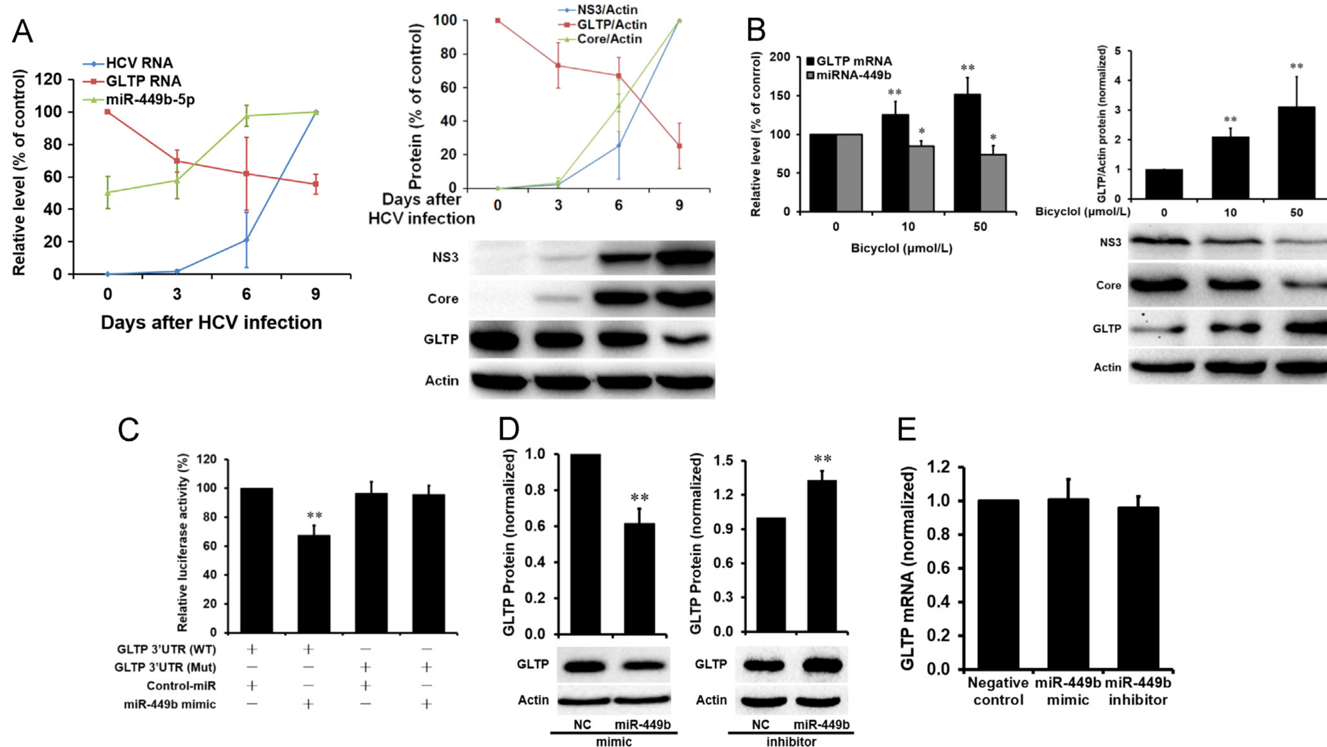

Figure 3 Bicyclol up-regulates intracellular GLTP *via* down-regulation of microRNA- 449b. (A) In the HCV-infected Huh7.5 cells, the level of *GLTP* mRNA (left) and protein (right) were decreased, while miR-449b (left) was increased. (B) In the HCV-infected Huh7.5 cells treated with bicyclol, the level of miR-449b (left) was decreased, and the level of *GLTP* mRNA (left) and protein (right) were increased; HCV NS3 and Core proteins (right) were decreased. Huh7.5 cells were infected with HCV and simultaneously treated with bicyclol. The RNA was extracted at 24 and 36 h for microRNA and mRNA analysis, respectively; and at 48 h, proteins were extracted for WB. (C) The miR-449b directly bound wild type (WT) *GLTP* 3′-UTR and decreased the relative intensity; however, it did not bound mutant (Mut) *GLTP* 3′-UTR. The 293T cells were co-transfected with recombinant pmirGLO plasmid containing wild type (WT) or mutant (Mut) *GLTP* 3′-UTR sequence for miR-449b binding and with miR-449b mimic using Lipofectamine 2000. A co-transfection with mimic negative control was as a control. The fluorescent intensity of firefly luciferase and renilla luciferase were detected stepwise in 24 h. While the miR-449b inhibited endogenetic GLTP expression, it did not influence the level of *GLTP* mRNA. The Huh7.5 cells were transfected with miR-449b mimic, or miR-449b inhibitor using Lipofectamine RNAiMAX. Intracellular proteins (D) and RNA (E) were detected in 48 h with WB and qRT-PCR, respectively.

GLTP 3′-UTR (Fig. 3C), but had no influence on the relative intensity of the vector with the mutant *GLTP* 3′-UTR (Fig. 3C), suggesting that the miR-449b could directly bind the wild type *GLTP* 3′-UTR. To further verify the regulatory effect of miR-449b on GLTP, qRT-PCR and WB analysis of GLTP was done in the Huh7.5 cells transfected with 50 nmol/L mimic or 100 nmol/L inhibitor of miR-449b. As expected, the GLTP protein expression was significantly decreased by the mimic of miR-449b (Fig. 3D,

left) and increased by the inhibitor of miR-449b (Fig. 3D, right), as compared with that in the control group (Fig. 3D), while the level of *GLTP* mRNA was not changed (Fig. 3E), suggesting that miR-449b binds the 3′-UTR of *GLTP* and thus influences the translation of *GLTP* mRNA, which decreases the protein level of GLTP with no influence on its mRNA level. These data suggested that bicyclol might up-regulate the GLTP expression *via* down-regulation of miR-449b. However, the detailed mechanism of how bicyclol regulate miR-449b remains to be illustrated; the minor increase of *GLTP* mRNA by bicyclol in the Huh7.5 cells (Fig. 3B) suggests that the binding of miR-449b to 3′-UTR might contribute to the stability of *GLTP* mRNA, or other unknown regulatory pathways might play a role.

3.4. GLTP is a novel host restrictive factor for HCV replication

We then analyzed whether alteration of intracellular GLTP level affects HCV replication *in vitro*. Thus, the GLTP high expression plasmid (GLTP+plasmid) was introduced into the Huh7.5 cells, followed by infecting the cells with HCV. As shown in Fig. 4A, intracellular HCV RNA (Fig. 4A, left) and proteins (Fig. 4A, right) in 72 h were significantly lower than that in cells transfected with the control plasmid (Fig. 4A), paralleled with an increase of the intracellular GLTP level (Fig. 4A, right). Transfection of the GLTP+plasmid with HA-tag or His-tag at C-terminus exhibited similar results (data not shown), indicating that fusing HA- or His-tag at the GLTP C-terminus did not affect its function. The results showed an inhibitory effect of GLTP on HCV replication. To view the effect from other angle, a silencing experiment was done as well, in which specific shRNA plasmid for GLTP was used to silence the endogenous GLTP in the Huh7.5 cells. The result showed that intracellular HCV RNA (Fig. 4B, left) and HCV proteins (Fig. 4B, right) elevated in 72 h after knocking-down of the expression of endogenous GLTP (Fig. 4B, right), suggesting an anti-HCV effect of GLTP in the viral replication cycle. Thus, GLTP might be a host restrictive factor for HCV replication.

We then valuated whether the anti-HCV activity of bicyclol is related to the up-regulation of GLTP in PHHs. The PHHs were infected with HCV and treated with bicyclol. The results showed that bicyclol decreased HCV RNA and increased *GLTP* mRNA in a dose-dependent manner (Fig. 4C, up) at day 7 post-infection. Bicyclol at 50 μmol/L exhibited an inhibitory effect on HCV replication equal to that of VX-950 at 0.1 μmol/L (Fig. 4C, up). VX-950 as an anti-HCV positive control showed no influence on the GLTP expression (Fig. 4C, up), though it was effective against HCV replication (Fig. 4C, up). The data were confirmed at protein level (Fig. 4C, down). The results suggest that bicyclol was also effective in PHHs and its inhibitory activity might be related to GLTP up-regulation.

To learn whether bicyclol inhibits HCV replication through GLTP, we did a rescue experiment. The HCV-infected Huh7.5 cells were transfected with shRNA plasmid for GLTP and then treated with bicyclol. The result showed that transfection with shRNA plasmid for GLTP abolished the anti-HCV activity of bicyclol at RNA (Fig. 4D, up) and protein (Fig. 4D, down) levels in 72 h, validating that cellular GLTP is essential for bicyclol's effect against HCV replication.

To investigate whether bicyclol up-regulates GLTP expression *in vivo*, we performed an experiment in mice. After 7-day treatment with bicyclol (25, 75 and 250 mg/kg, orally, bid for 7 days), the liver *Gltp* mRNA (Fig. 4E, up) and protein (Fig. 4E, down) were increased in a dose-dependent manner, while VX-950 treatment (375 mg/kg, orally, Bid for 7 days) did not change the GLTP expression (Fig. 4E). The mice body weight did not change at day 4 and 7 post drug treatment (Supporting Information Fig. S3), suggesting that bicyclol could up-regulate GLTP *in vivo* with no toxicity.

3.5. GLTP restricts HCV replication by interrupting the interaction between VAP-A and NS5A

The cellular vesicle-associated membrane protein-associated protein-A (VAP-A) is an important constituent directly interacting with HCV NS5A and forming a replicative complex essential for HCV RNA replication[12,13]. The binding of GLTP on the VAP-A has been reported previously[14], but the biological significance is not clear. To confirm the binding between GLTP and VAP-A, we designed and conducted a co-IP experiment. The results showed that GLTP bound VAP-A and the band was featured with the His-tag signal (Fig. 5A, lane 3), which was consistent with the previous report with the GST pull-down assay[14]. With a similar design, we investigated whether alteration of GLTP level manipulates the interaction between VAP-A and NS5A. Indeed, NS5A bound VAP-A in the co-IP assays (Fig. 5B), agreeing with the previous reports[12,13]. A high level of GLTP reduced the amount of NS5A bound with VAP-A (Fig. 5B, lane 3), and a low level of GLTP increased the binding of NS5A on VAP-A (Fig. 5B, lane 4). NS5A didn't directly bind GLTP in the co-IP assays (Fig. 5C), and was no longer part of the GLTP/VAP-A complex in the presence of GLTP (Fig. 5C), because GLTP bound VAP-A in a preferential manner (Fig. 5C). Furthermore, the alteration of GLTP expression by exogenous expression (Fig. 5D, left) or shRNA (Fig. 5D, right) plasmid did not influence the level of VAP-A in the cells. The results suggested that the GLTP does interrupt the interaction between VAP-A and NS5A, causing blockage of the attaching of NS5A to VAP-A through its competitive and preferential binding with VAP-A.

To learn how GLTP interrupts VAP-A/NS5A interaction, we constructed a group of tryptophan (Try or W) site-mutant of GLTP (Fig. 5E, left), in which the W85F and W142F mutants do not influence GLTP's bio-function, while the W96F mutant partially and W96A mutant completely cause loss of the bio-function for acquisition and release of glycolipid through selectively altering the stacking between the sugar and indole rings[15,16]. Firstly, we tested their anti-HCV activities *in vitro*. Huh7.5 cells were transfected with those plasmids and then infected with HCV. Intracellular proteins were analyzed in 72 h. Fig. 5E showed that the W85F and W142F mutants of GLTPs showed anti-HCV activity comparable to that of the wild type (WT) GLTP, the W96F mutant of GLTP reduced its anti-HCV activity by 44.6–62.5%, and the W96A mutant of GLTP had no activity against HCV (Fig. 5E, left). It appears that the anti-HCV activity of GLTPs is related to their biological function. To further validate the hypothesis, we constructed another set of GLTP mutants (Fig. 5F, left), of which the 33-35 amino acids (FFD) of GLTP plays a key role in binding with VAP-A, but does not influence GLTP function[14]. The AA mutant at the 33–34 site partially bound VAP-A, while the AAA mutant at the 33–35 sites completely lost its capacity of binding with VAP-A (Fig. 5F, right). The results agreed with the previous report[14]. We then tested the anti-HCV activity of those mutants. Huh7.5 cells were transfected with the plasmids and infected with HCV. The results showed that the AA

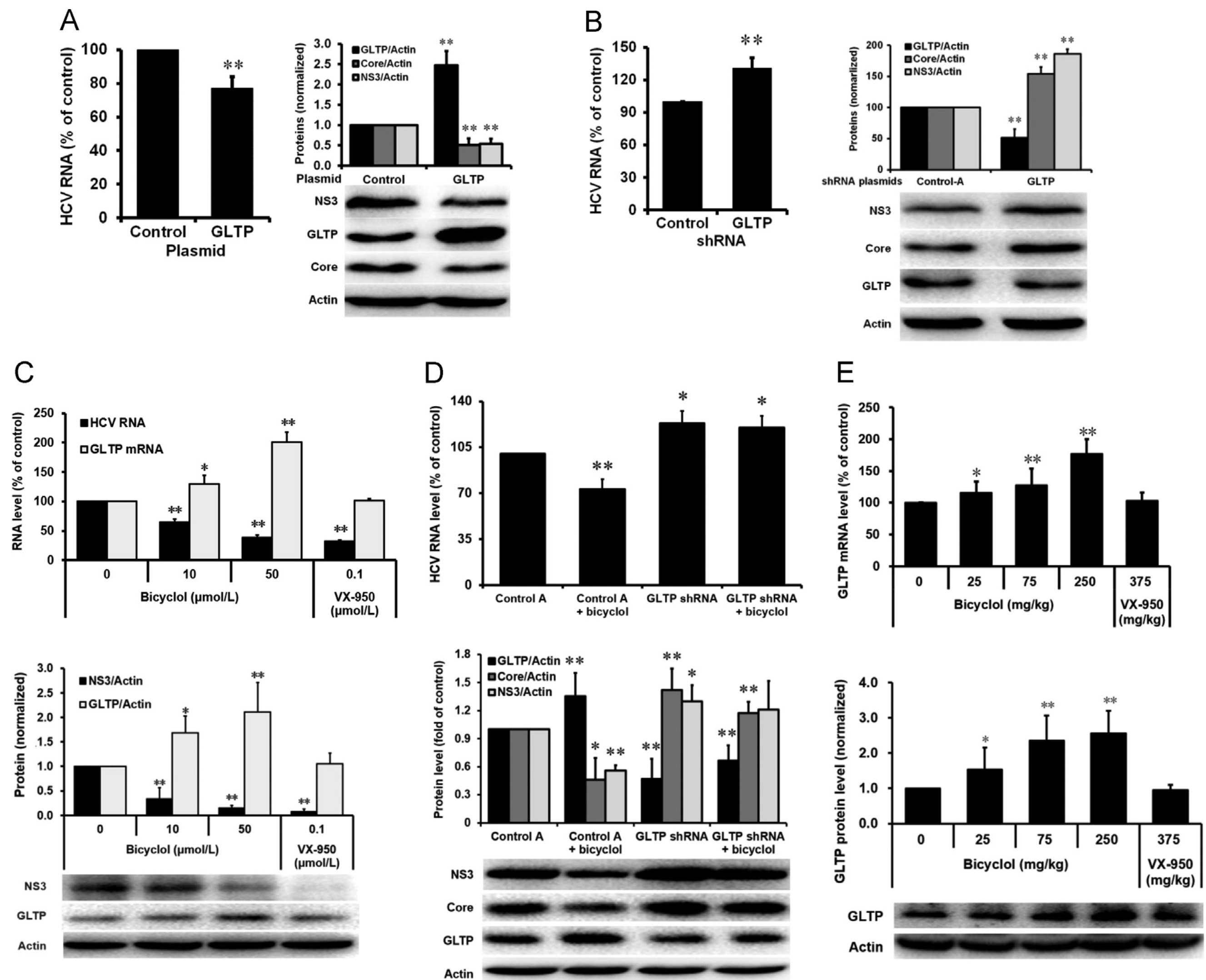

Figure 4 GLTP is a novel host restrictive factor for HCV replication. HCV replication was negatively correlated with the level of GLTP. Huh7.5 cells were transfected with GLTP expression plasmid (A) or with shRNA plasmid for GLTP (B), a pcDNA3.1(+) vector and shRNA plasmid-A were used as negative controls, respectively. Six hours later, the cells were infected with HCV. Intracellular RNA and proteins were detected with qRT-PCR and WB in 72 h ($n = 3$; $^{*}P < 0.05$, and $^{**}P < 0.01$ *vs.* plasmid control). (C) Primary human hepatocytes were infected with HCV (2000 IU/cell) and were simultaneously treated with bicyclol or VX-950. Intracellular RNAs (up) were quantified with qRT-PCR, and proteins (down) were analyzed with WB in 7 days ($n = 3$; $^{*}P < 0.05$, and $^{**}P < 0.01$ *vs.* solvent control). (D) Bicyclol inhibited HCV replication *via* GLTP. Huh7.5 cells were infected with HCV (45 IU/cell) and then transfected with shRNA plasmid for GLTP or plasmid control, six hours post infection, the cells were treated with bicyclol (20 μmol/L) or solvent control. Intracellular HCV RNA (up) and proteins (down) were detected in 72 h ($n = 3$, $^{*}P < 0.05$, and $^{**}P < 0.01$ *vs.* plasmid controls). (E) Mice were randomly divided into five groups with 10 mice (5 male and 5 female) in each group, and administered intragastrically with bicyclol, VX-950 or solvent control twice a day for 7 days. The mice were sacrificed and the liver tissues were collected at day 7. Liver total RNAs (up) were quantified with qRT-PCR and proteins (down) were analyzed with WB. The protein bands presented in the figure showed the results of a representative experiment. The data presented are the mean ± standard deviation. The Student's *t*-test was used.

mutant at the 33–34 site showed a decreased anti-HCV activity, while the AAA mutant at the 33–35 site had no inhibitory activity on HCV (Fig. 5F, left), suggesting that the GLTP's binding capacity to VAP-A is essential for its anti-HCV effect. For further confirmation, a co-IP experiment was performed with the tryptophan (W) site-mutant GLTPs. The results showed that the GLTP with W85F and W142F mutation strongly interrupted VAP-A/NS5A interaction, and resulted in a decrease of NS5A (Fig. 5E, right), while the GLTP with W96A mutation didn't interrupted VAP-A/NS5A interaction (Fig. 5E, right). The results were consistent with their anti-HCV activities (Fig. 5E, left) and further suggested that the GLTP restricts HCV replication through interrupting the interaction between VAP-A and NS5A.

3.6. Bicyclol synergistically inhibits HCV replication with known anti-HCV drugs and is effective on HCV variants resistant to DAAs

Considering the unique anti-HCV mechanism of bicyclol, we speculated that bicyclol might be a good antiviral agent in

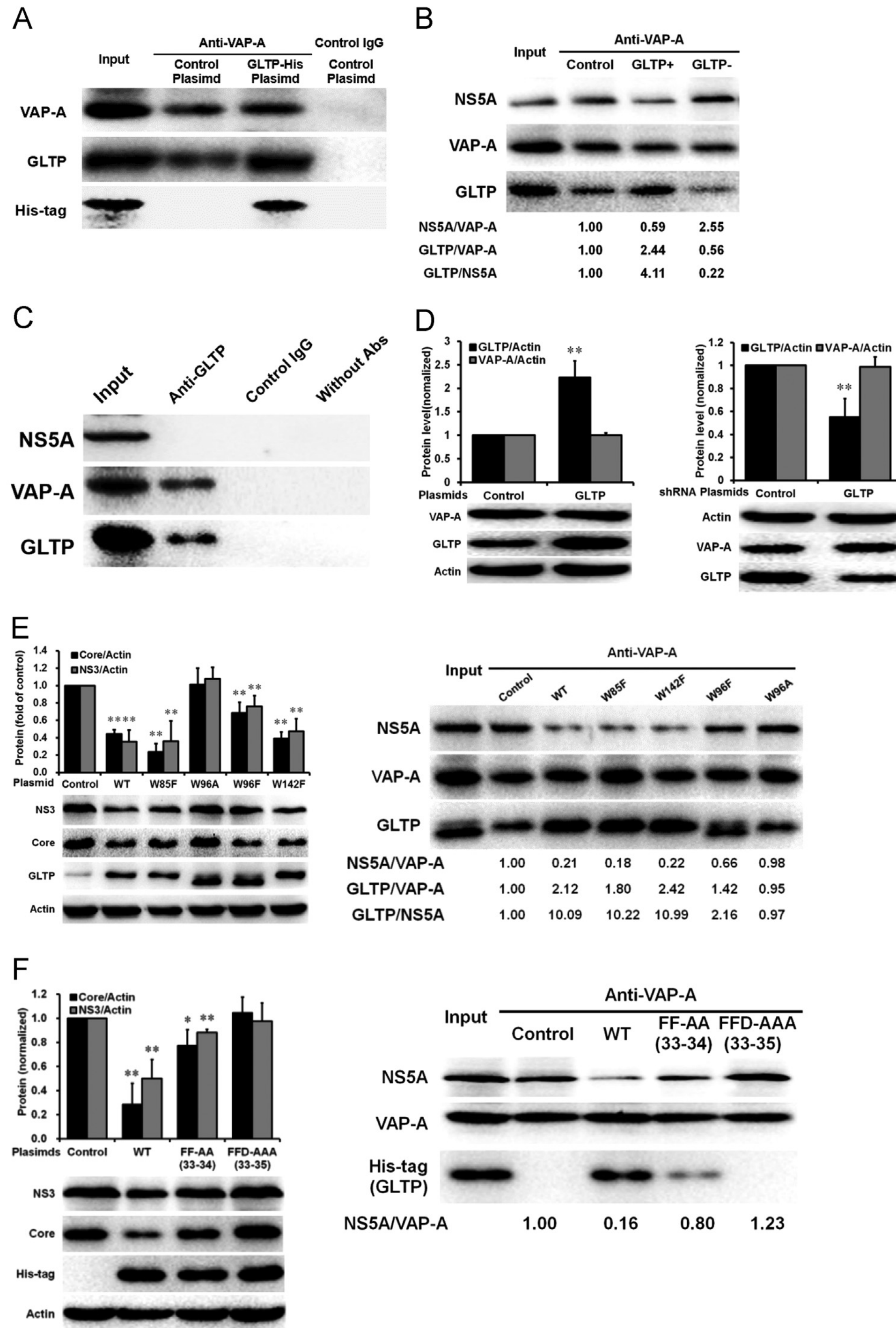

Figure 5 GLTP restricts HCV replication by interrupting the interaction between VAP-A and NS5A. (A) The GLTP directly bound VAP-A in a co-IP assay. The protein VAP-A was as the bait protein, and the binding was confirmed by detecting His-tag of the GLTP-His. (B) The level of GLTP negatively affected the level of NS5A bound to VAP-A in the co-IP assay. The protein VAP-A was as the bait protein, and the Huh7.5 cells transfected with expression plasmid or shRNA plasmid for GLTP were used. (C) GLTP had no direct interaction with NS5A in the co-IP assay. The protein GLTP served as the bait protein. (D) Changing the level of GLTP did not influence the level of intracellular VAP-A protein. The Huh7.5 cells were transfected with the expression plasmid or shRNA plasmid for GLTP; intracellular proteins were detected in 72 h by WB ($n = 3$; $^{**}P < 0.01$ *vs.* control). The anti-HCV activity and a co-IP assay for mutants of GLTP. The Huh7.5 cells were transfected with GLTP of wild type or W site-directed mutant (E) or amino acid 33-35 mutant (F), and infected with HCV (45 IU/cell). After 72 h incubation, intracellular proteins were extracted and detected with WB (left, $n = 3$; $^{*}P < 0.05$ *and* $^{**}P < 0.01$ *vs.* control). For co-IP assay, the protein VAP-A was as the bait protein (right). The detail method of the co-IP assay was in the Material and Methods. The experiments were carried out for over 3 times. The protein bands presented in the figure showed the results of a representative experiment. The data presented are the mean $\pm$ standard deviation of three independent experiments and the Student's *t*-test was used in A–F.

Table 1 Synergism of bicyclol plus drugs against HCV.

Drug	EC_{50} (μmol/L)	CI values[a]				DRI[b]	
		EC_{50}	EC_{75}	EC_{90}	EC_{95}	Bi	Drugs
Bicyclol (Bi)	30.678	–	–	–	–	–	–
Telaprevir (Tel)	0.025	–	–	–	–	–	–
Interferon-α-2b (IFN)	0.727 (U/mL)	–	–	–	–	–	–
Simeprevir (Sim)	0.023	–	–	–	–	–	–
Sofosbuvir (Sof)	0.106	–	–	–	–	–	–
Combination Bi+Tel	13.771 + 0.014	0.997	0.789	0.701	0.678	2.2	1.8
Combination Bi+IFN	13.039 + 0.261 (U/mL)	0.784	0.607	0.490	0.432	2.4	2.8
Combination Bi+Sim	13.640 + 0.007	0.738	0.685	0.709	0.766	2.2	3.4
Combination Bi+Sof	13.154 + 0.026	0.676	0.685	0.748	0.829	2.3	4.0

–not detected.

[a]CIs were evaluated by calculating by the Chou-Talalay method using CompuSyn version 1.0.

[b]Folds of dose reduction allowed for each drug caused by synergism at a given effect level (*e.g.*, the dose reduction index (DRI) values at EC_{50} effect level).

Table 2 The inhibitory activity of bicyclol in HCV mutants resistant to DAAs.

Drug	EC_{50} (μmol/L)[a]				Fold of resistance[b]
	WT	A156T	D168V	S282T	
Bicyclol	19.34 ± 5.66	20.59 ± 1.15	19.08 ± 2.42	18.67 ± 3.08	1.06–0.97
Telaprevir	0.015 ± 0.005	0.734 ± 0.348	–	–	49
Simeprevir	0.006 ± 0.003	–	0.485 ± 0.184	–	81
Sofosbuvir	0.076 ± 0.040	–	–	0.510 ± 0.137	7

–not detected.

[a]EC_{50} was calculated with Reed-Muench method after HCV RNA were detected with qRT-PCR. The data presented are mean ± standard deviation (n = 3).

[b]The valve of EC_{50} for mutant *vs.* EC_{50} for wild type (WT).

combination with the known anti-HCV drugs. To confirm this, HCV-infected Huh7.5 cells were treated with bicyclol, plus one of the approved DAAs drugs or interferon (IFN). The results showed that bicyclol inhibited HCV replication in a synergistic pattern when used together with telaprevir or simeprevir or sofosbuvir or IFN, showing a combination index (CI) less than 1.0 at EC_{50}–EC_{95} concentration, and a dose-reduction index (DRI) lager than 1.0 (Table 1). The results were validated at protein level (Supporting Information Fig. S4). It suggests that bicyclol might be a new member of the regimen for HCV treatment, if validated in patients.

Emergence of drug-resistant HCV causes DAAs treatment failure in clinic[17,18]. For this sake, we tested the anti-HCV effect of bicyclol in the HCV variants that are resistant to DAAs. HCV variant D168V[19] and A156T[20] are the most frequently seen clinical mutants resistant to NS3/4A protease inhibitors (such as simeprevir and telaprevir), and S282T mutant is resistant to sofosbuvir[21]. Our results (Table 2) showed that those mutants were resistant to the mentioned DAAs as previously reported[19–21], but showed a high sensitivity to bicyclol, similar to that of the WT HCV (Table 2). The results were evidenced at the HCV protein level (Supporting Information Fig. S5). It suggests that bicyclol may be a good drug candidate in treating patients infected with HCV mutants resistant to DAAs.

4. Discussion

Over the past decades, many TCM-derived drugs did show substantial anti-HCV effect through regulating host factors involving innate immunity[22]. Although the anti-HCV efficacy of the TCM therapeutic regimens is not as potent as the current DAAs regimens, the mode of action of the TCM is of great importance for understanding their immune regulatory roles, which might be the reason causing spontaneous clearance of HCV. The present study showed for the first time that bicyclol could create a host restricting-effect against HCV. As about 30% of HCV-infected individuals showed spontaneously clearance of the virus through host mechanism[23,24], it is possible to eliminate HCV from the body *via* promoting immunity-associated host factors. Here, we showed that induction of glycolipid transfer protein expression by bicyclol caused spontaneous interruption of HCV replication. Importantly, the GLTP-based molecular link between innate immunity and TCM might be of general interest to those who work with TCM worldwide[25,26].

GLTP is a small soluble cytosolic protein and its biological role is not fully understood. The physiological function of GLTP may relate to the transports of sphingoid- and glycerol-based glycolipids in the vesicular system[27]. Further results showed that GLTP may transport glycolipids from the Golgi to the plasma membranes[27–29], and could directly interact with VAP-A which might

Figure 6 The proposed diagram for the interaction among the GLTP, NS5A and VAP-A and the anti-HCV mechanism of bicyclol. (A) The proposed diagram for the interaction among the GLTP, NS5A and VAP-A. In the major sperm protein domain of the VAP-A, the FFAT domain of GLTP bind VAP-A in competition with the domain 3 of NS5A, through the partially overlapped binding sites. (B) The key amino acids of the VAP-A in interacting with the GLTP or NS5A. GLTP binds with the conserved amino acids 45K, 47T, 87K and 118K (yellow) of the VAP-A; and NS5A goes for the key amino acids Q6, M89, V90, Q91, D116, L119 and V122 (red) of the VAP-A. The amino acids in the box show the three domains in the VAP-A. (C) Schematic diagram of bicyclol's anti-HCV mechanism through up-regulation of GLTP. Treatment of the HCV-infected Huh7.5 cells with bicyclol deceased the level of miR-449b and thus up-regulated the level of GLTP. The up-regulated GLTP preferentially bound host VAP-A in competition with the HCV NS5A, causing an interruption of the interaction between VAP-A and HCV NS5A which is essential for HCV RNA replication, and thus inhibited HCV replication.

be important as the VAP-A could assist HCV replication by forming a complex with HCV NS5A[14]. Using bicyclol as a chemical probe, the present study discovered GLTP to be a new host restrictive factor for HCV replication. It seems that GLTP restricts HCV replication mainly through its binding with VAP-A which interrupts the complex formation between VAP-A and NS5A.

HCV NS5A is important for HCV propagation and is one of the three key targets to develop new anti-HCV drugs, but its function in the viral life cycle remains to be clarified[30]. HCV NS5A binds

host VAP-A to form a replicative complex, which is required for an efficient replication of HCV genomic RNA[12]. Previous study showed that disruption of the interaction between NS5A and VAP-A by mutation or de-phosphorylation of NS5A suppresses HCV RNA replication[13]. We showed here that the GLTP interrupted the interaction between VAP-A and NS5A, and the low level of NS5A/VAP-A complex restricted HCV RNA replication (Fig. 5E and F). In addition, it has been reported that VAP-A itself provides assistance to HCV replication in the complex, with the molecular mechanism unknown[12,13]. It could be another anti-HCV action for GLTP in its binding with VAP-A. The present study showed that GLTP did not interact with the viral NS5A directly, and the NS5A was not able to bind the complex of GLTP/VAP-A, suggesting that GLTP competitively binds VAP-A in a preferential manner and thus prevents NS5A from its attachment on the VAP-A. More investigation is needed to illustrate the detailed mechanism of GLTP in its interaction with VAP-A or NS5A/VAP-A complex.

The VAP-A consists of an N-terminal major sperm protein (MSP) domain (aa.1–124), a linker region with a coiled coil domain (aa.158–194), and a C-terminal transmembrane helix tail (aa.223–242) anchored at the endoplasmic reticulum (Fig. 6A). Seven amino acids (in red) in the MSP domain bind intrinsically the unstructured domain 3 of NS5A (aa.359–447) (Fig. 6B)[31]. And highly conserved residues (in yellow) in the MSP domain directly interact with two phenylalanines in an acidic tract-like motif (FFAT domain of GLTP), which contains the 32-38 amino acids PFFDCLG in GLTP[14,32]. This interaction forms an electro-positive face (in blue) on the MSP domain (Fig. 6A). However, the binding sites of GLTP in the MSP domain partially overlaps with that of NS5A (Fig. 6B)[33], and the binding affinity between GLTP and VAP-A is stronger than that between NS5A and VAP-A with algorithm[33]. Thus, bicyclol increases the level of GLTP, which preferentially binds host VAP-A in competition with the HCV NS5A, and therefore blocks the interaction between VAP-A and NS5A, causing a decrease of the HCV replicative complex. This action improves the intracellular immunity, which produces an interruption of HCV RNA duplication and spontaneously clears HCV (Fig. 6C).

Up-regulated GLTP by bicyclol is considerably safe. Our *in vitro* experiments showed a normal cell growth pattern in the Huh7.5 cells with either up-regulated- or silenced-GLTP expression, consisting with previous reports in the HeLa, HEK-293 and A549 cells[34]. In addition, after up-regulation of liver GLTP, the mice grew normally. To the best of our knowledge there was no report showing adverse effects in the GLTP gene knock-in or -out animals. The GLTP expression was regulated by miR-449b, which is one of the isoforms of miR-449 and has its function related to cell cycle, differentiation and apoptosis[35]. Also important is the good safety of bicyclol in human at the dose used in the study. Three months treatment with bicyclol caused no damage to liver, kidney and bone marrow function in the patients. In fact, bicyclol decreased blood level of liver enzymes, consistent with its activity of liver protection[3,6]. Bicyclol was approved by the Chinese FDA as a hepatoprotective drug, and in the past decade bicyclol has been proved to be effective and safe in clinic[1]. To show the HCV-restricting effect of bicyclol *in vivo*, results from a clinical trial in patients with chronic hepatitis C are presented, showing a 95% clearance of HCV by bicyclol. Also evidenced is the good safety of bicyclol in human. Three months treatment with bicyclol caused no damage to liver, kidney and bone marrow function in the patients. In fact, bicyclol decreased blood level of liver enzymes, consistent with its protective effect on liver function[3]. The data suggest that up-regulation of GLTP by bicyclol is relatively safe.

DAAs and fixed-dose combinations have shown high cure rate in treating HCV infected patients[36,37]. However, drug resistance-caused treatment failure remains to be a challenge[17]. To overcome the viral drug-resistance, host-targeting agents (HTAs) working through cellular antiviral mechanisms to clear the virus remains attractive[38]. With respect to the DAAs, HTAs might have advantages to treat HCV infection, such as a high genetic barrier to drug-resistance and a pan-genotypic antiviral activity. Combination of HTAs with DAAs caused a synergistic antiviral effect in clinic[39] and reduced a rate of drug resistant mutation [40]. We showed that bicyclol inhibited HCV replication through up-regulating host restrictive factor GLTP, and could create a synergistic inhibition in combination with known DAAs or IFN. As bicyclol is a hepatoprotective agent in clinic[1,3,5], it might be a good candidate for HCV-infected patients who have liver problems and are not recommended for the exciting DAAs[41,42]. Also, as the anti-HCV mechanism of bicyclol is very different from that of DAAs, though its efficacy is not so strong as DAA's, it could be a member in future for the treatment of CHC, in combination with known DAAs.

Previous clinical observations showed that HCV RNA level decreased in hepatitis C patients after bicyclol mono-therapy[1,6]. In these previous Phase IV clinical trials, the hepatitis C patients were treated with bicyclol after failure to the interferon-alpha plus ribavirin therapy. To validate the anti-HCV effect of bicyclol, the present clinical trial only enrolled the hepatitis C patients who were not treated with antiviral agents before. Results showed that the blood HCV viral load went down after treating with bicyclol, consistent with the previous observations. Liver enzymes declined significantly as well, agreeing with previous reports[1,6]. The maximum concentration of bicyclol is about 123.87 μg/L (0.32 μmol/L) in healthy adult plasma after oral administration of bicyclol at 50 mg, tid for 7 days[43], and our *in vitro* results also showed anti-HCV activity under this concentration (Fig. 1). The clinical data strongly support our *in vitro* results.

In summary, GLTP appears to be a novel host HCV-restrictive factor in hepatocytes. Up-regulation of GLTP by bicyclol reduces the complex level of VAP-A/NS5A, interrupts HCV replication and thus decreases HCV level in the host. Furthermore, the results might provide an antiviral molecular mechanism in TCM, in the view of host innate immunity against HCV.

Acknowledgments

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Appendix A. Supporting information

Supporting data associated with this article can be found in the online version at https://doi.org/10.1016/j.apsb.2019.01.013.

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