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Davioud-Charvet^{2,3*} and Thomas Pietschmann^{1*} A Plant-Derived
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A Plant-Derived Flavonoid Inhibits Entry of All HCV Genotypes into Human Hepatocytes

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Abbreviations

Hepatitis C Virus (HCV), Scavenger receptor class B type I (SR-BI), 2'-C-methyladenosine (2'CMA)

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Abstract

Background & Aims: Interferon-based therapies for hepatitis C virus (HCV) infection are limited by side effects and incomplete response rates, particularly among transplant recipients. We screened a library of plant-derived small molecules to identify HCV inhibitors with novel mechanisms.

Methods: We isolated phenolic compounds from *Marrubium peregrinum* L (Lamiaceae). Replication of HCV RNA, virus production, and cell entry were monitored using replicons and infectious HCV. Inhibition of HCV was measured in hepatoma cells and primary human hepatocytes using luciferase reporter gene assays, core ELISAs, or infectivity titration. We tested the bioavailability of the compound in mice.

Results: We identified a flavonoid, ladanein (BJ486K), with unreported antiviral activity and established its oral bioavailability in mice. Natural and synthetic BJ486K inhibited a post-attachment entry step, but not RNA replication or assembly; its inhibitory concentration 50% was 2.5 micromolar. BJ486K was effective against all major HCV genotypes, including a variant that is resistant to an entry inhibitor; it prevented infection of primary human hepatocytes. Combined administration of BJ486K and cyclosporine A had a synergistic effect in inhibition of HCV infection.

Conclusions: BJ486K has oral bioavailability and interferes with entry of HCV into cultured human hepatocytes. It synergizes with cyclosporine A to inhibit HCV infection. Its inhibitory effects are independent of HCV genotype, including a variant that is resistant to an entry inhibitor against SR-BI. Flavonoid derivatives might therefore be developed as components of combination therapies, because they are potent, broadly active, inhibitors of HCV entry that could prevent graft reinfection after liver transplantation.

Key words

Antiviral therapy, liver transplantation, entry inhibitor, drug

Introduction

End stage liver disease associated with chronic HCV infection is a leading indication for liver transplantation¹. Unfortunately, reinfection of the donor organ is universal. In fact, 20-25% of graft recipients develop cirrhosis within 5 years and the survival of HCV-positive patients is lower compared to HCV-negative recipients^{2, 3}. Current antiviral treatment of chronic HCV infection is based on PEG-IFN- α and ribavirin and is limited by side effects and suboptimal response rates particularly in difficult-to-treat transplant patients⁴. First generation of directly acting antivirals which target the viral NS3-4A protease have improved treatment options. However, resistance development⁵ and viral genotype-specific efficacy⁶ of these drugs necessitate alternative antiviral treatment options. Therefore, new therapeutics that eliminate chronic virus replication, prevent progression to end stage liver disease or preclude reinfection of the donor organ in HCV-associated orthotopic liver transplantation could provide a substantial benefit.

HCV is a highly variable, hepatotropic RNA virus that establishes a chronic infection in the majority of cases. Viral strains are grouped into six major genotypes that differ from each other by more than 30% at the nucleotide level⁷. The variability of HCV permits immune evasion and thus facilitates viral persistence. It is also a challenge for development of specific antiviral therapies active against all viral genotypes and for prevention of drug resistance.

Recently, cell culture systems reproducing the complete replication cycle of HCV have been developed⁸ and utilized to define novel drug targets and to identify and characterize new antiviral compounds. These studies, for instance, revealed an essential role of four host derived proteins, the scavenger receptor class B type I (SR-BI), CD81, claudin-1 (CLDN1) and occludin (OCLN) for productive cell entry by HCV⁹. In turn, interference with the early steps of HCV infection, which are essential for spread and maintenance of HCV infection, has emerged as attractive target for development for novel therapeutic options¹⁰. In parallel, several natural flavonoids including silibinins, silymarin and naringenin, which interfere with

HCV propagation by targeting the viral polymerase¹¹ or by alternative mechanisms likely also involving host-derived factors, have recently been identified¹²⁻¹⁴ and thus have highlighted compounds with flavonoid scaffold as attractive molecules for HCV drug development. Importantly, HCV liver graft reinfection was recently successfully prevented by silibinin monotherapy¹⁵. These promising advances prompted us to screen various polyphenolic compounds and glycosides for novel inhibitors of HCV infection.

Material and Methods

Synthesis of BJ486K

The total synthesis of BJ486K was developed according to the known procedure¹⁶ with slight modifications. The full details of the total synthesis will be described in a further manuscript, as part of a physicochemistry analysis. The chemical analysis was in agreement with that of the natural compound, ladanein (Supplementary Figure S2).

Cell Culture

Huh7-Lunet/CD81 cells, a derivative of Huh-7 cells highly permissive for HCV RNA replication and infection¹⁷, were cultured in Dulbecco's modified Eagle medium (DMEM, Invitrogen) with 10% fetal bovine serum (FCS Gold, PAA, Coelbe, Germany), 1x non-essential amino acids (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and 100 IU/ml penicillin (Invitrogen), 2 mM L-glutamine (Invitrogen) and 750 µg/ml geneticin (Carl Roth, Germany).

In vitro transcription and electroporation of Huh7-Lunet/CD81 cells

In vitro transcripts were generated and transfected using electroporation as described recently¹⁸.

Luciferase infection assay

Huh7-Lunet/CD81 cells were infected with reporter virus particles essentially as described recently¹⁸. Briefly, for inoculation with *firefly* luciferase reporter virus particles (Luc-Jc1¹⁸) and *renilla* reporter viruses (JcR-2a¹⁹ and GT1-6, this study), Huh7-Lunet/CD81 cells were seeded at a density of 6×10⁴ cells per well of a 12-well plate or 6.6×10³ cells per well of a 96-well plate 24h prior to inoculation. If not stated otherwise, the virus particles were pretreated with the inhibitors for 1h at 37°C. Cells were inoculated for 4h at 37°C, viral inoculum was replaced by fresh culture fluid and infection was quantified 48h or 72h after virus inoculation using luciferase assays. To this end, cells were washed once with PBS and

in the case of the Luc-Jc1 infected cells, lysed directly on the plate with 350 µl of cold lysis buffer (0.1% Triton-X100, 25 mM glycylglycine, 15 mM MgSO₄, 4 mM EGTA and 1 mM dithiothreitol, pH 7.8) and after addition of the luciferase solution (200 µM luciferin, 25 mM glycylglycine, pH8) measured for 20s in a luminometer (Lumat LB9507, Berthold, Freiburg, Germany). Cells infected with the renilla reporter virus were lysed in 200 µl lysis buffer (Promega, Mannheim, Germany) and after addition of the luciferase substrate (1 µM of coelenterazin (P.J.K., Kleinblittersdorf, Germany) in PBS) measured for 1s in a luminometer (Lumat LB9507, Berthold, Freiburg, Germany). The indicated EC₅₀ values were determined by using Sigma Plot software (Systat Inc; San Jose; CA).

Freeze and thaw lysis of HCV-transfected cells

Huh7-Lunet/CD81 cells were transfected with Jc1 RNA and seeded on a 6-well plate. Compounds were added 4h later. At 48h after transfection, cell culture supernatant was harvested and used for measuring the extracellular core amount. Cells were washed once with PBS, scraped and centrifuged for 5 min at 1000×g. Cell pellets were resuspended in 500 µl fresh culture fluid and subjected to three cycles of freeze and thaw using liquid nitrogen and a 37°C thermo block. Samples were then centrifuged at 10,000×g for 10 min to remove cell debris and the core amount was measured (intracellular core protein) using the ARCHITECT HCV Core AG test (Abbott, Wiesbaden, Germany) according to the instructions of the manufacturer.

Cytotoxicity assay

Cytotoxicity was measured using the CytoTox-Glo cytotoxicity assay (Promega, Mannheim, Germany) as described by the manufacturer employing a plate luminometer Centro XS LB960 (Berthold, Freiburg, Germany) according to the manufacturer's instructions.

Determination of antiviral activity against early steps of HCV cell entry

JcR-2a virus particles were incubated with Huh7-Lunet/CD81 cells seeded as described above for 1h at 4°C to synchronize cell entry. Various inhibitors including 50 µg/ml heparin (Sigma-Aldrich), CD81-specific antibody JS-81 (2 µg/ml; Becton Dickinson), ITX 5061 (10

μM) or BJ486K (20 μM), were added during virus inoculation (protocol 1) or at indicated time points after virus inoculation (protocol 2-8). After 4h inhibitor treatment, medium was changed to fresh culture fluid without inhibitors and the cells were lysed 48h after inoculation as described above. Efficiency of infection was determined by luciferase assay using a tube luminometer (Lumat LB9507, Berthold, Freiburg, Germany).

Immunohistochemical staining and virus titration

Virus titers were measured using the limiting dilution assay as described recently²⁰. Virus titers (50% tissue culture infective dose [TCID₅₀/ml]) were calculated based on the method of Spearman and Kärber^{21, 22}.

Infection of primary human hepatocytes

Normal-appearing liver tissue from patients seronegative for HCV, hepatitis B virus, and human immunodeficiency virus (HIV) was obtained from adult patients undergoing partial hepatectomy for the therapy of metastases. Experimental procedures were carried out in accordance with French Laws and Regulations. Hepatocytes were isolated by a two-step perfusion technique and maintained in primary culture as described previously²³. Cells were inoculated with Con1/C3-HCV²⁴ grown in Huh-7.5.1 cells at a multiplicity of infection of 0.1 in the presence of dimethylsulfoxide as carrier control, or else increasing concentrations of BJ486K. Three days later, infectivity titers in culture supernatants as well as HCV RNA amounts were determined by focus-formation assay or qRT-PCR analysis respectively as previously described^{23, 25}.

Results

Identification of a flavonoid with antiviral activity against HCV

To identify novel bioactive molecules with antiviral activity, a bio-guided fractionation was performed starting from a *Lamiaceae* plant, a *Marrubium* species reported to be rich in flavone aglycones, flavone glycosides, coumaroylated flavone glycosides and acteoside-related phenylethanoids²⁶. Natural phenolic compounds were isolated from plant extracts of *Marrubium peregrinum* L. (*Lamiaceae*) (Supplementary Figure S1) and anti-HCV activity of these extracts was evaluated by inoculation of the highly permissive human hepatoma cell line Huh7-Lunet/CD81¹⁷ with firefly luciferase reporter viruses based on the intragenotypic genotype 2a chimera Jc1 in the presence of the compounds (Luc-Jc1¹⁸; Figure 1). Among all tested extracts, LF-LA19 and LF-MP03, which comprise large quantities of two closely related flavonoids (tricine-7-glucoride and ladanein, respectively), inhibited HCV cell entry and/or RNA replication by more than 99% or 99.9%, respectively. To confirm that ladanein, the dominant flavonoid present in extract MP03, is exclusively responsible for the observed antiviral activity against HCV, we synthesized ladanein and compared the antiviral activity of the synthetic compound, designated BJ486K, with the one from the natural source. The structure and purity of synthetic BJ486K was confirmed by NMR spectroscopy, mass-spectrometry, elemental analysis (Supplementary Figure S2) and X-ray radiocrystallography (data not shown). Both MP03 as well as BJ486K inhibited infection and/or RNA replication by the genotype 2a luciferase reporter virus in a dose-dependent fashion (Figure 2A). The effective dose 50% (EC50; Figure 2A) and toxic dose 50% (TC50; Figure 2B) were comparable between MP03 (EC50: 2.79 μ M; TC50: 90.03 μ M) and BJ486K (EC50: 2.54 μ M; TC50: 98.04 μ M), providing evidence that BJ486K is the active component within the plant-derived extract MP03. These data demonstrate that the flavone BJ486K efficiently inhibits HCV entry, RNA-translation and/or replication.

BJ486K inhibits HCV cell entry but not RNA replication or virus production

Our screening assay does not distinguish which step of the HCV replication cycle is affected by a given compound. Therefore, to characterize the antiviral effect of BJ486K, we next assessed the impact of BJ486K on the steps of HCV RNA translation and replication. To this end, we transfected Huh7-Lunet/CD81 cells with a subgenomic JFH1 luciferase replicon and monitored RNA replication 72h later by luciferase assays. As control we incubated the cells with increasing doses of the NS3-4A protease inhibitor boceprevir and the polymerase inhibitor 2'-C-methyladenosine (2'CMA). As shown in Figure 3A, BJ486K affected neither RNA translation nor replication of HCV RNA as luciferase activity was not changed up to a dose of 10 μ M.

To determine whether BJ486K affects virus assembly or release we transfected Huh7-Lunet/CD81 cells with Jc1 RNA and quantified intra- and extracellular levels of core protein 48h after transfection (Figure 3B). As expected, both quinidine and the grapefruit flavonoid naringenin, two assembly inhibitors recently identified using the JFH1-based infection system^{14, 27}, selectively reduced extracellular levels of core protein by more than 100- or ca. 5-fold, respectively, thus confirming their interference with HCV virus production. In contrast, BJ486K reduced neither intracellular nor extracellular core protein levels up to a dose of 20 μ M, which is 10-fold higher than the determined EC50 value (Figure 2). Collectively these data indicate that different flavonoids can prevent HCV propagation by distinct mechanisms and that BJ486K does not impede HCV RNA translation, RNA replication and virus production. In agreement with this conclusion, addition of BJ486K only interfered with HCV infection when the drug was present during virus inoculation, whereas addition of the compound before or after virus inoculation did not preclude productive cell entry and subsequent RNA replication (Figure 3C).

BJ486K interferes with a post-attachment step of HCV cell entry

To resolve which step of HCV cell entry is inhibited by BJ486K, we assessed its antiviral activity when administered at different time points during the early phase of infection. To this end, Huh7-Lunet/CD81 cells were inoculated for one hour at 4°C with a reporter virus

prepared in the absence of drugs. At this temperature, virus particles bind to the cell surface but do not efficiently enter, thus permitting a rather synchronous infection when the inoculum is removed and cells are shifted to 37°C. A selection of HCV entry inhibitors which prevent infection by different mechanisms or BJ486K were added during inoculation, directly afterwards or at different intervals shortly thereafter. To attain maximal sensitivity we used a novel monocistronic renilla luciferase reporter virus based on the intragenotypic genotype 2a chimera Jc1 which is designated JcR-2a and that was described recently¹⁹ (Figure 4). As expected, heparin which is known to impede HCV cell attachment¹⁸, only interfered with HCV infection when present already during inoculation (Figure 4). In contrast, BJ486K like CD81-specific antibodies, potently inhibited entry not only when present during inoculation but also when added directly after virus binding (>90% inhibition). In fact, administration of the drug 20 minutes post virus attachment still inhibited ca. 80% of HCV infection, clearly indicating that BJ486K blocks a post-binding step of the HCV entry pathway. The kinetics of BJ486K-mediated blockade of HCV cell entry were comparable to CD81-specific antibodies and also to ITX5061, a clinical stage compound that is thought to interfere with HCV infection through inhibiting viral interaction with SR-BI²⁸.

BJ486K is not affected by viral resistance against a SR-BI-targeting entry inhibitor, inhibits HCV of all major genotypes and impedes infection of primary human hepatocytes

Syder et al. recently reported that a specific mutation within the HCV envelope protein E2 (G451R) that is known to reduce viral dependency on SR-BI for cell entry²⁹ at the same time reduces the susceptibility of HCV towards inhibition by ITX5061²⁸. These data indicate that decrease of SR-BI receptor dependence by HCV is a possible viral escape mechanism from inhibition by SR-BI-targeting antivirals. Therefore, to explore whether this viral resistance mechanism also compromises the antiviral activity of BJ486K we compared the antiviral activity of the two compounds against wild type and the mutant virus carrying the G451R

exchange within E2. In line with the report by Syder et al., the aforementioned mutation rendered HCV resistant to inhibition by ITX5061 (Figure 5A). In contrast, the antiviral activity of BJ486K was not changed by this mutation (Figure 5B), indicating that reduced SR-BI dependence does not confer resistance towards entry inhibition by BJ486K.

Efficacy of current HCV treatment is substantially influenced by viral genotype. More specifically, IFN-based therapy regimens attain sustained viral response rates of ca. 80% in patients infected with genotype 2 and 3 viruses whereas response rates among patients chronically infected with genotype 1 reach only about 50%³⁰. Moreover, directly targeted antivirals in late stage clinical development vary in efficacy depending on the viral genotype. Therefore, we determined the antiviral activity of BJ486K towards HCV particles from different genotypes. To be able to resolve different response rates among these viruses with highest resolution, we created a set of novel reporter virus constructs based on the JcR-2a backbone (Figure 4). Importantly, antiviral activity of BJ486K against cell culture derived particles from genotype 1a, 1b, 2b, 3a, 4a, 5a and 6a was comparable and showed an EC50 below 4.2 μ M indicating that BJ486K inhibits HCV cell entry independently of viral genotype or subtype (Figure 5C and Supplementary Table 1).

Although human hepatocellular carcinoma derived cell lines sustain the entire HCV replication cycle and are a well-accepted model for HCV infection studies, these cells nevertheless functionally differ from primary human hepatocytes. Therefore, to confirm the antiviral activity of BJ486K in a more natural system, primary human hepatocytes were inoculated in the presence or absence of BJ486K with a cell culture derived genotype 1b virus (Con1/C3; ²⁴). As shown in Figure 5D, Con1/C3 infection of primary human hepatocytes was inhibited by BJ486K in a dose-dependent fashion with an EC50 value of ca. 10 μ M.

BJ486K exerts synergistic antiviral activity with Cyclosporine A (CsA) and prevents productive infection of differentiated human hepatoma cells. Next, we investigated if combination of CsA, an inhibitor of HCV RNA-replication *in vitro* and *in vivo* and also an immunosuppressive drug used in the HCV post-transplant setting, with BJ486K would

increase antiviral potency. To this end, we challenged Huh7-Lunet/CD81 cells differentiated by DMSO-treatment³¹ with the highly infectious Jc1 chimera in the presence of CsA, BJ486K or a combination of both drugs (Figure 6). In this quiescent, growth arrested cell culture we monitored virus production for 12 days after inoculation to find out if drug treatment had completely eliminated virus infection. The applied dose of CsA was not sufficient to prevent infection of the cells and resulted in only about 5 to 10-fold lower levels of infectious virus 12 days after inoculation (Figure 6B). In contrast, BJ486K reduced infection almost down to background levels (Figure 6C). Remarkably, combination of both drugs completely eliminated infection as is evident from absence of detectable infectious virus at days 6, 9 and 12 post inoculation (Figure 6D). To obtain a more quantitative measure for the combined antiviral effect of BJ486K and CsA, we inoculated naïve Huh7-Lunet/CD81 cells with JcR-2a virus together with varying doses of both drugs (Figure 7A). Importantly, addition of 1 μ M of BJ486K decreased the EC₅₀ of CsA by more than 10-fold (left panel) and vice versa, as little as 100 ng/mL of CsA decreased the EC₅₀ of BJ486K by ca. 3-fold to ca. 400 nM. To further analyze the drug-drug-interaction, we examined the combined antiviral activity over a broad range of concentrations for both inhibitors (7.81-2000 ng/mL for CsA and 0.31-10 μ M for BJ486K). Employing the method of Prichard and Shipman³² we observed that specifically low doses of both drugs resulted in an antiviral activity above the expected value indicating that BJ486K and CsA synergistically inhibited HCV infection (Figure 7B). Of note, first pharmacokinetic data of BJ486K administered to mice indicate bioavailability of the drug and peak plasma levels of 329 nM after a single oral dose of 0.25 mg/kg (Supplementary Figure S3). Collectively, these results indicate that combination of these inhibitors dramatically increases antiviral potency which is sufficient to prevent infection of cultured cells with a high dose of infectious HCV.

Discussion

Screening a library of plant-derived extracts, we identified a flavone with potent antiviral activity against HCV. Further mechanistic studies indicated that the natural compound as well as the synthetic molecule designated BJ486K prevent HCV cell entry by blocking a post attachment step. Antiviral activity was confirmed in primary human hepatocytes, it was independent of viral genotype and was not affected by a recently described viral resistance mechanism that decreases efficacy of SR-BI-targeting compounds. Importantly, combination of BJ486K with CsA synergistically increased antiviral potency and prevented successful infection of cultured cells with a high dose of infectious HCV. Recently, Firipi et al. compared use of CsA with tacrolimus in HCV liver transplant recipients undergoing antiviral therapy and observed a modest drop of viral RNA and increased antiviral response to PEG/RBV in the CsA-based regiment of immunosuppression³³. Although the study was small, these data nevertheless suggested some antiviral benefit to use of CsA. Notably, a more recent retrospective comparison between CsA and tacrolimus in the HCV-transplant setting involving more than 8,000 patients reported a better long-term outcome for patients receiving tacrolimus³⁴. Although the molecular mechanism underlying this result is unclear it argues that antiviral effects of CsA are outweighed by beneficial characteristics of tacrolimus in the long term. Nevertheless, transient regiments in the early phase after liver transplantation involving CsA and including cell entry inhibitors – like for instance BJ486K – may prevent HCV recurrence. Subsequently, immunosuppression could be switched to tacrolimus for further improving chances of graft survival.

HCV cell entry is a complex multistep process that involves at least four cell type specific entry factors (CD81, SR-B1, CLDN1 and OCLN). Since ectopic expression of these proteins in human and non-human cells renders these permissive for HCV infection, it seems that additional important cellular proteins are highly conserved and ubiquitously expressed. Collectively these in part cell type specific host components mediate virus attachment and endocytosis via clathrin coated vesicles. Ultimately, a low pH-induced fusion mechanism

permits viral escape from endosomes and access of the viral genome to the cytoplasm for subsequent steps of HCV RNA translation and RNA replication. While the precise role of entry factors during infection is incompletely understood, interference with the usage of these proteins by HCV has emerged as an attractive approach for development of novel therapeutics. In fact, antibodies specifically targeting CD81 have been shown to prevent infection of humanized mice when administered before viral challenge³⁵. These encouraging data suggest that modalities preventing virus cell entry could be used in the future to prevent reinfection of liver graft after HCV-associated liver transplantation. Moreover, also in chronically infected patients entry inhibitors could complement future therapeutic regimens by preventing *de novo* infection of host cells, thus contributing to viral clearance.

Our data indicate that BJ486K prevents HCV life cycle steps subsequent to virus binding and prior to viral RNA translation and RNA replication. Thus, receptor interactions, virus endocytosis, or membrane fusion could be inhibited. Considering the antiviral activity of BJ486K against all HCV genotypes, we analysed if it inhibits infection by alternative viruses unrelated to HCV (Supplementary Figure 4). Interestingly, BJ486K interfered with infection by VSV and HIV-1, but not CoxV and AdV5. These data indicate that BJ486K has a broad-spectrum antiviral activity inhibiting infection by enveloped virus particles (HCV, HIV-1, VSV), but not by virus particles without lipid membrane (CoxV, AdV5). Given these results it is unlikely that BJ486K interferes with HCV infection by preventing specific HCV-receptor interactions. Instead this compound likely precludes entry steps that are shared or reasonably conserved between HCV, HIV-1 and VSV but fundamentally different from CoxV and AdV entry mechanisms. In this regard virus-cell fusion which is mediated by viral fusion proteins operating through shared mechanisms among enveloped viruses and modulating membrane properties of host and viral membranes may be a candidate target for BJ486K. It will be interesting to find out if BJ486K inhibits cell fusion of HCV and other viruses, which chemical properties may confer this inhibitory property and whether this could be further exploited for development of broadly active antiviral entry inhibitors.

Collectively, our data highlight a novel flavone with potent and broad-spectrum antiviral activity against HCV. These findings should encourage further development of flavone-derived compounds as attractive pan-genotype entry inhibitors for treatment of HCV.

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Figure legends

Figure 1: Screening of plant-derived compounds for antiviral activity against HCV. A schematic drawing of the Luc-Jc1 reporter virus genome¹⁸ used for the screening is depicted at the top. Huh7-Lunet/CD81 cells were inoculated over night with Luc-Jc1-containing preparations in the presence or absence of given extracts. HCV infection efficiency was determined 72h post inoculation using luciferase assays and is expressed as % of DMSO control. Mean values of quadruplicates including the standard deviation are shown.

Figure 2: Antiviral activity of BJ486K. (A) Huh7-Lunet/CD81 cells were inoculated for 48h with Luc-Jc1 virus particles in the presence of increasing doses of MP03 or BJ486K, respectively. HCV infection and replication efficiency was determined 48h post inoculation using luciferase assays and is expressed as % of cells treated with solvent alone. Mean values of three independent experiments each with quadruplicate measurements including the standard deviation are shown. (B) Huh7-Lunet/CD81 cells were cultured for 48h in the presence of increasing doses of MP03 or BJ486K, respectively. Cell viability was determined using a commercial cytotoxicity assay (CytoToxGlo; Promega, Germany) and is expressed relative to cells treated with DMSO. Mean values of three independent experiments each with quadruplicate measurements including the standard deviation are shown.

Figure 3: BJ486K selectively inhibits HCV cell entry. (A) Huh7-Lunet/CD81 cells were transfected with the subgenomic luciferase replicon depicted at the top. Drugs were added 4h post transfection and the level of HCV RNA translation and RNA replication was quantified by luciferase assays 72h post transfection. Mean values of three independent experiments each with quadruplicate measurements including the standard deviation are shown. (B) Cells were transfected with the Jc1 chimera depicted above and drugs were added 4h later. HCV virus release in the presence or absence of given compounds was

determined by quantification of intracellular and extracellular core protein levels using a core-specific ELISA. Mean values of three independent experiments with single measurements including the error range are given. (C) Huh7-Lunet/CD81 cells were inoculated with Luc-Jc1 reporter viruses prepared in the absence of drugs. BJ486K was added to the cells only prior to inoculation (black), only during inoculation (grey) or selectively directly after inoculation (white) as schematically depicted at the top. Infectivity was determined 72h later by luciferase assays and compared to DMSO-treated controls. Mean values of three independent experiments each with quadruplicate measurements including the standard deviation are shown.

Figure 4: BJ486K inhibits cell entry post virus attachment.

The design of the JcR-2a reporter virus construct is given at the top. Huh7-Lunet/CD81 cells were inoculated with concentrated JcR-2a particles at 4°C for 60 minutes. Subsequently, unbound virus particles were washed away and cells were shifted to 37°C to permit synchronized cell entry. According to the 8 experimental protocols depicted in the middle, various cell entry inhibitors including heparin (50 µg/ml), ITX5061 (10 µM), CD81-specific antibody JS-81 (2 µg/ml) and BJ486K (20 µM) were either present during inoculation (protocol1) or were added at different time points after inoculation (protocols 2 to 8). In each case, inhibitor was present for 4h (dashed line) and was then washed away. Efficiency of cell entry was determined 48h post inoculation by determination of luciferase activity and is expressed relative to control infections conducted in the presence of solvent alone (DMSO). Mean values of three independent experiments each with quadruplicate measurements including the standard deviation are shown.

Figure 5: BJ486K is not affected by viral resistance towards a SR-BI-targeting compound, inhibits HCV of different genotypes and prevents infection of primary human hepatocytes. Huh7-Lunet/CD81 cells were inoculated with Luc-Jc1 or Luc-

Jc1/E2/G451R reporter viruses in the presence of increasing doses of (A) ITX 5061 or (B) BJ486K. Cells were lysed 72h later. Luciferase activity was determined and is expressed relative to control infections conducted in the presence of DMSO. Mean values of four independent experiments each with quadruplicate measurements including the standard deviation are shown. (C) Huh7-Lunet/CD81 cells were inoculated with genotype 1a, 1b, 2b, 3a, 4a, 5a and 6a chimeric reporter viruses in the presence of increasing doses of BJ486K. Luciferase activity was determined 72h later and is expressed relative to infections in the presence of DMSO. Mean values of three independent experiments, each with triplicate measurements, are given. (D) Primary human hepatocytes were inoculated with Con1/C3 in the presence of DMSO or increasing concentrations of BJ486K. The inhibition of HCV infection was assessed by focus-formation assay (black) or by qRT-PCR analysis (grey) respectively and is expressed as % inhibition relative to DMSO-treated cells. Mean values of duplicate measurements including the error range are given.

Figure 6: Combination therapy of BJ486K and CsA prevents infection of differentiated human hepatoma cells. Huh7-Lunet/CD81 cells were differentiated by treatment with 1% DMSO as described recently³¹. Subsequently cells were challenged with Jc1 in the presence of DMSO (A), CsA (B), BJ486K (C), or a combination of CsA and BJ486K (D). Culture fluids of the inoculated cells were collected at 3, 6, 9 and 12 days post inoculation and the infectivity of released virus particles was determined by a limiting dilution assay (n.d: not detectable). Dotted line depicts the measurement background of the assay.

Figure 7: Synergistic antiviral activity of BJ486K and CsA. (A) Combined effects of CsA on BJ486K and vice versa were analysed by inoculation of Huh7-Lunet/CD81 cells with JcR-2a virus in the presence of given drugs and determination of luciferase activity 72h later. Mean values of three independent experiments each conducted in triplicates including the standard deviations are given. Data are normalized for values determined in mock treated controls. (B) Antiviral effect of CsA and BJ486K drug combinations in Huh7-Lunet/CD81

cells. The dose ranges of CsA and BJ486K are given. Shading indicates different ranges above or below the expected value. A volume below the zero plane indicates an antagonistic, a volume above the plane a synergistic effect.

A **B**

A **B** **C**

binding
(4°C)

entry and replication
(37 °C)

inhibitor

A **B** **C** **D**

A **B** **C** **D**

A **B**

Supplementary material

Material and Methods:

Preparation of plant extracts and isolation of ladanein

Ladanein (MP03) was extracted according to the reported procedures^{1, 2}. Briefly, natural phenolic compounds (Supplementary Figure S1) were isolated from plant extracts of *Marrubium peregrinum* L. (*Lamiaceae*), following extraction of dried, powdered flowered aerial parts with dichloromethane at room temperature, evaporation and lyophilization. The extract was submitted to gel filtration by Sephadex LH-20 in the biofractionation process. Isolated compounds were purified over Silicagel 60H columns or further Sephadex LH-20 as described previously². The compounds were identified by NMR spectroscopy (Supplementary Figure S2), X-ray radiocrystallography and mass spectrometry. Details regarding the biofractionation process are available upon request.

Plasmids

The plasmids pFK-Luc-Jc1 and pFK-Jc1 encoding the intragenotypic 2a/2a chimeric virus Jc1 with or without firefly luciferase reporter gene^{3, 4} as well as the plasmid pFKi389RLuc2aCore-3'-Jc1 (⁵ referred to as JcR-2a) have been described. Briefly, JcR-2a is a monocistronic reporter virus derived from the Jc1 chimera⁴ that expresses a *Renilla* luciferase (RLuc) that is fused N-terminally with the 16 N-terminal residues of the core protein. C-terminally RLuc is connected to the Jc1 open reading frame through the foot-and-mouth disease virus (FMDV) 2A peptide coding region which liberates the reporter from the downstream HCV proteins. This construct was used to create infectious monocistronic RLuc reporter virus genomes for all six major genotypes. Briefly, chimeric HCV open reading frames encoding core, E1, E2, p7 and NS2 of the GT1a isolate H77C^{6, 7}, the GT1b isolate J4^{8, 9}, the GT2b isolate J8⁸, the GT3a isolate S52¹⁰, the GT4a isolate ED43⁶, the GT5a isolate SA13¹¹, or the GT6a isolate HK6a⁸, respectively, followed by JFH1-derived NS3 to NS5B genes were cloned downstream of the R2a-reportergene cassette. The resulting novel

monocistronic *Renilla* luciferase reportervirus genomes were designated H77c/1a/R2a, J4/1b/R2a, J8/2b/R2a, S52/3a/R2a, ED43/4a/R2a, SA13/5a/R2a, HK6a/6a/R2a.

Infection assays with HIV, vesicular stomatitis virus, adenovirus and coxsackievirus

Env-deleted HIV-1-GFP reporter viruses, which had been pseudotyped with the R5 envelope from YU-2, were incubated with the indicated concentrations of either BJ486K or MP03 (Supplementary Figure 4) for 1h at 37°C, in principle as reported¹². Briefly, TZM-bl cells were challenged with these viruses overnight, washed and subsequently cultivated under standard conditions. Three days post-infection the percentage of infected, GFP-positive cells was quantified by flow cytometry. Values obtained for solvent-treated controls were set to 100%. Shown are arithmetic means $\pm$ SD (n=3) from one of three independent experiments. The indicated EC₅₀ values were determined by using Prism software (GraphPad, San Diego, CA). Replication competent group B coxsackievirus (CoxV) and adenovirus type 5 (AdV5), or a GFP-tagged vesicular stomatitis virus (VSV-GFP) were incubated with the indicated concentrations of BJ486K for 1h at 37°C prior to infection of Vero-CH, A549 or Huh-7.5 cells, respectively. Virus titers were analysed using limiting dilution assays. In brief, cells were inoculated with serially diluted virus preparations with six replicate wells of a 96-well plate per dilution. Cells were fixed with 3% paraformaldehyde 48h to 72h after infection and in the case of CoxV and AdV5 infection stained with coomassie solution (0.25% Coomassie brilliant blue R-250, 45% methanol, 10% acetic acid) to identify infected wells. In case of VSV infections, we used a recombinant vesicular stomatitis virus expressing enhanced green fluorescent protein designated VSV*M_Q¹³. This virus, kindly provided by Gert Zimmer, carries four attenuating mutations in the M protein and expresses GFP from an additional transcriptional unit located between G and L. VSV*M_Q infected cells were identified by detection of GFP-autofluorescence using a Olympus IX-81 microscope. Virus titers (50% tissue culture infective dose [TCID₅₀/ml]) were calculated based on the method of Spearman and Kärber^{14, 15}.

Evaluation of bioavailability in mice.

Mice (24 in total) were treated either orally or intravenously with a single dose of 0.25 mg/kg of the inhibitor (solved in saline/10% cremophor EL). At different time points after inhibitor administration (5 min, 15 min, 30 min, 60 min, 120 min or 180 min) two mice from each group were sacrificed and the blood was centrifuged to obtain the plasma, which was stored at -80°C until further examination.

400 µl of each plasma sample was mixed with equal amount of a 2% trifluoroacetic acid and loaded on a SPE Strata C18 column (equilibrated with 1 ml methanol and 1 ml 1% trifluoroacetic acid) for solid phase extraction of the plasma. After several washing steps (1% trifluoroacetic acid and water), the samples were eluted with acetonitrile and dried. The remaining sample was resolved in 25 µl acetonitrile:water (ratio 1:1). Samples were further analyzed with a LC-MS/MS (triple quadrupole mass spectrometer LCMS 8030 Shimadzu), which was calibrated at the day of measurement. Plasma drug concentrations versus time curves were plotted for each route. Bioavailability was calculated by dividing the area under curve (AUC) for oral route by the AUC for intravenous route.

Supplementary Table 1: Antiviral activity of BJ486K across all major HCV genotypes

Figure S1: Structures of polyphenolic compounds analysed in this work. All compounds depicted were screened for antiviral activity against HCV using the screening system described in Figure 1. The short name of the extract containing the respective compound as dominant species is given along with the chemical name of the compound.

Figure S2: ¹H and ¹³C NMR spectroscopic data and elemental analysis detailing the characterization of the synthetic ladanein BJ486K. The exact structure of BJ486K was determined according to the NMR spectroscopy. ¹H (top) and ¹³C (bottom) NMR spectra were recorded at room temperature on a Bruker Avance 300 spectrometer (¹H at 300 MHz and ¹³C at 75 MHz). Chemical shifts are referenced to the solvent signal (¹H NMR, δ 7.26

ppm; ^{13}C NMR, δ 77.0 ppm). The chemical analysis was in agreement with that of the natural ladanein². Characteristic resonance signals, proton and carbon assignments of the synthetic BJ486K are shown in both spectra as well as the values of the NMR chemical shifts and data of the elemental analysis.

Figure S3: Oral bioavailability of BJ486K in mice. A single dose of 0.25 mg/kg of BJ486K was administered orally (A) or through the intravenous route (B). Plasma levels of BJ486K were determined 5, 15, 30, 60, 120 and 180 minutes post administration. Mean values of two animals including the standard errors are given.

Figure S4: Differential antiviral effect of BJ486K on selected enveloped and non-enveloped viruses. (A) Influence of BJ486K and MP03 on HIV was determined as described in materials and methods. (B) The influence of BJ486K on enveloped vesicular stomatitis viruses and non-enveloped group B coxsackievirus (CoxV) and adenovirus type 5 (AdV5) was determined using limiting dilution assays.

Supplementary Literature:

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Table 1. Antiviral activity of BJ486K across all major HCV genotypes

HCV isolate	EC ₅₀ [μM]	EC ₉₀ [μM]
H77c (GT1a)	2.46	11.64
J4 (GT1b)	2.35	9.29
J8 (GT2b)	4.14	8.42
S52 (GT3a)	2.89	10.65
ED43 (GT4a)	0.74	1.78
SA13 (GT5a)	1.39	5.33
HK6a (GT6a)	0.75	2.39

LF-MP09
Arenarioside

LF-LA08
Acteoside

LF-LA09
2''-Acetylacteoside

LF-LA12
Isoacteoside

LF-MP08
Ballotretoside

LF-LA11
Forsythoside B

LF-LA10
Calceolarioside E

LF-LA13
Cistanoside F

LF-MP01
Marrubinin

LF-LA05
Mussaenoside

LF-LA06
8-epi-loganin

LF-LA07
Geniposide

LF-MP03
Ladanein

LF-LA14
Apigenin-7-glucuronide

LF-MKKA
Keayanidine A

LF-LA15
Luteolol-7-glucuronide

LF-LA16
Luteolol-7-diglucuronide

LF-LA17
Clerodendrin

LF-LA18
3''-Acetylclerodendrin

LF-LA19
Tricin-7-glucuronide

LF-MP06
Terriflorin

A **B**

A **B**