MK-571, a cysteinyl leukotriene receptor-1 antagonist, inhibits hepatitis C virus (HCV) replication

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Abbreviations: ABC: ATP-binding cassette; HCV: hepatitis C virus; SGR: subgenomic replicon; GT: genotype; EC₅₀: effective concentration 50%
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Abstract:

The quinoline MK-571 is the most commonly used inhibitor of multidrug resistance protein-1 (MRP-1) but was originally developed as a cysteinyl leukotriene receptor 1 (CysLTR1) antagonist. While studying the modulatory effect of MRP-1 on anti-hepatitis C virus (HCV) direct acting-antivirals (DAA) efficiency, we observed an unexpected anti-HCV effect of compound MK-571 alone. This anti-HCV activity was characterized in Huh7.5 cells stably harboring a subgenomic genotype 1b replicon. A dose-dependent decrease of HCV RNA levels was observed upon MK-571 administration, with an EC$_{50}$ of 9±0.3 µM and a maximum HCV RNA level reduction of approximately 1 Log$_{10}$. MK-571 also reduced the replication of the HCV full-length J6/JFH1 model in a dose-dependent manner. However, probenecid and apigenin homodimer (APN), two specific inhibitors of MRP-1, had no effect on HCV replication. In contrast, the CysLTR1 antagonists SR2640 increased HCV-SGR RNA levels in a dose-dependent manner, with a maximum increase of 10-fold. In addition, a combination of natural CysLTR1 agonist (LTD4) or antagonists (zafirlukast, cinalukast, and SR2640) with MK-571 completely reversed its antiviral effect, suggesting its anti-HCV activity is related to CysLTR1 rather to MRP-1 inhibition. In conclusion, we showed that MK-571 inhibits HCV replication in hepatoma cell cultures by acting as a CysLTR1 receptor antagonist, thus unraveling a new host-virus interaction in the HCV life cycle.
Hepatitis C virus (HCV) is a blood-borne, single-stranded, positive-sense RNA virus belonging to the Hepacivirus genus of the Flaviviridae family. During the HCV life cycle, the viral genome of approximately 9,600 nucleotides is translated into a polyprotein that is subsequently cleaved by cellular and viral proteases into 3 structural proteins (E1, E2 and core) and 7 nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) (1). Nonstructural proteins NS3, NS4A, NS4B, NS5A and NS5B associate with host proteins to form the viral replication machinery, while p7 and NS2 are essential for infectious virus production (2).

Worldwide, 71 million people are estimated to be infected with HCV, representing approximately 1% of the world population, most of whom have chronic liver disease. Chronic HCV infection causes almost 400,000 deaths annually, principally from the complications of cirrhosis or hepatocellular carcinoma (3). Highly efficacious and well-tolerated combinations of direct-acting antiviral (DAA) drugs have revolutionized HCV treatment. Infection cure rates higher than 95% can now be achieved, with a measurable impact on HCV-related morbidity and mortality (4). Four main classes of DAAs are commercially available, including NS3/4A protease inhibitors, NS5A protein inhibitors, nucleoside analogs, and non-nucleoside inhibitors of the NS5B RNA polymerase (5).

Despite the spectacular virological results of current anti-HCV therapies, several issues remain. In patients who fail to achieve a cure of the infection, HCV variants carrying resistance-associated substitutions (RAS) on their genome, i.e. substitutions that confer reduced susceptibility to the administered drugs, are generally selected (6). Their long-term persistence after treatment raises issues as to subsequent retreatment.
Although the global rate of treatment failure is low with current DAA combinations, the absolute number of patients requiring retreatment is high. This number will further increase due to the large number of patients who will be treated, in the context of the World Health Organization endeavor to eliminate HCV as a major public health threat by 2030 (3). Importantly, some regions of the world (e.g. central Africa, South-East Asia) harbor unusual subtypes of known genotypes that are inherently resistant to commonly administered DAAs (7, 8). In addition, the high cost of last-generation DAA regimens limits access to care in low-income areas, while the management of special patient groups, such as those with advanced liver disease or renal failure, may be problematic with current drugs.

Multidrug resistance (MDR), i.e. cell ability to acquire drug resistance, is mainly mediated by the overexpression of membrane drug transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP) or multidrug resistance protein-1 (MRP-1) which belong to the ATP-binding cassette (ABC) transporter superfamily (9) (10). These transporters influence drug pharmacokinetics, particularly their distribution, thereby modifying their concentrations in cells (11). Drug–drug interactions may occur at the transporter level and modulate drug efficacy and/or toxicity (12). Functional interactions between anti-HCV DAAs and ABC transporters have been reported (4, 13). Indeed, almost all of the approved HCV inhibitors, including sofosbuvir, daclatasvir, ledipasvir, velpatasvir, voxilaprevir, paritaprevir, dasabuvir, glecaprevir and pibrentasvir are substrates and/or inhibitors of at least one ABC transporter (4, 14).

To investigate the involvement of ABC transporters in the efflux of HCV protease inhibitors, we had tested the in vitro anti-HCV activity of the first-generation, first-wave NS3-4A protease inhibitor telaprevir, alone or in combination with LY335979 (15), KO143 (16), or MK-571 (17, 18), inhibitors of P-gp, BCRP and MRP-1, respectively. In the
control experiments, we observed an unexpected antiviral effect of MK-571 alone, a result that prompted us to characterize the anti-HCV activity of this compound and identify its target.

In addition to MRP-1, MK-571 has been reported to target cysteinyi leukotriene receptor-1 (CysLTR1) (18). Cysteinyi leukotrienes (LT) include LTC4, LTD4 and LTE4. They are lipid mediators derived from arachidonic acid (AA) via the 5-lipoxygenase pathway (19, 20). Their biological effects are mediated by distinct cysteinyi leukotrien receptor (CysLTR) belonging to the G protein-coupled receptor family. CysLTRs have been reported to be involved in inflammation, shock, allergic reactions, plasma extravasations and liver injury (21-23). CysLTR1 has been extensively studied and selective antagonists have been developed (24-26). They include montelukast (Singulair®, Merck, New York, USA) and zafirlukast (Accolate®, AstraZeneka, London, UK), which are used for the treatment of bronchial asthma and allergic rhinitis (27).

In the present study, based on our serendipitous discovery of the anti-HCV activity of MK-571, we characterized the antiviral activity of this compound in cellular HCV models and identified its target.
MATERIALS AND METHODS

Drugs

MK-571, MK-886, zileuton, probenecid, cinalukast and zafirlukast were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA), while telaprevir was purchased from AGV discovery (Montpellier, France). APN was kindly provided by Dr Attilio Di Pietro (Institut de Biochimie et Chimie des Protéines, Lyon, France). SR2640 was purchased from Tocris (Bristol, UK).

HCV plasmid

Plasmid I389-Neo/NS3-3’/5.1, that contains a neomycin resistance gene and an HCV genotype 1b subgenome, was kindly provided by Prof. Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany). This plasmid has been transfected into Huh7.5 cells to generate Huh7.5-SGR cells stably harboring an HCV genotype 1b subgenomic replicon (SGR).

Cell Culture

Human hepatoma-derived Huh7.5 cells (Apath LLC) were cultured in complete Dulbecco’s modified Eagle medium (DMEM, ThermoFisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% fetal bovine serum, 50 IU/ml penicillin, 100 µg/ml streptomycin, and 0.1 µg/ml amphotericin B (ThermoFisher Scientific). Huh7.5-SGR cells were cultured in the same medium supplemented with 0.5 mg/ml of
G418 (ThermoFisher Scientific) to eliminate Huh7.5 cells that do not harbor the subgenomic replicon.

Assessment of anti-HCV activity in the subgenomic replicon model

Huh7.5 cells stably harboring an HCV genotype 1b bicistronic replicon ($I_{389-Ne/oNS3-3'}/5.1$) were seeded at the density of 5,000 cells/well in a 96-well plate. The cells were treated for 48 h with the tested compounds diluted in complete DMEM containing 1% DMSO with 0.5mg/ml of G418. Total RNA was extracted using the RNeasy 96 kit (QIAgen, Hilden, Germany). HCV- SGR RNA levels were measured by RT-qPCR using the Taqman technology with HCV-specific primers (forward primer 5'-'CGC CCA AAC CAG AAT ACG A-3' and reverse primer 5'-AGA TAG TAC ACC CTT TTG CCA GAT G-3') and qPCR probe (5'-6-FAM-CAA TGT GTC AGT CGG-TAMRA-3'). The data were analyzed with the 2^{-ΔΔCT} method, with all samples normalized to GAPDH mRNA. All experiments were performed in triplicate. HCV-SGR RNA relative quantities were plotted against compound concentrations and fitted with a four-parameter logistic curve with SigmaPlot v11 software. EC_{50}s were determined from the curves.

Assessment of antiviral activity in the HCV infectious model

Huh7.5 cells were incubated 24 hours before infection. Then, cells were infected with HCV (J6/JFH1 strain) at an MOI of 0.2 during 8 hours in presence of drugs. HCV-infected cells were washed with PBS and incubated for 48 hours in fresh medium containing the drugs. Then, luciferase activity was measured and plotted against compound concentrations.
Cytotoxicity assay

Assessment of the compounds cytotoxicity was performed with The CellTiter 96® AQueous One (Promega), according to the manufacturer instructions. Huh7.5 cells were seeded at a density of 5,000 cells/well in a 96-well plate and incubated 24h before addition of increasing concentration of the indicated compounds. After 48 hours, cells were incubated 4 hours in presence of The CellTiter 96® AQueous One Solution Reagent and absorbance was measured at 490nm.

Statistical analysis

Statistical analyses were performed using SigmaPlot software. Statistics were calculated using t-test analysis of variance. P values below 0.05 were considered statistically significant.
RESULTS

MK-571 inhibits HCV replication

With the goal to assess the effect of inhibiting ABC transporter (e.g. MRP-1) efflux on the antiviral activity of HCV protease inhibitor telaprevir, we measured HCV RNA levels in Huh7.5 cells stably harboring a genotype 1b HCV-SGR treated with 1 µM of telaprevir in combination with 50 µM of MK-571. In our control experiments with MK-571 in the absence of telaprevir, a 12-fold decrease of HCV RNA levels was observed, suggesting that MK-571 bears anti-HCV activity. The antiviral effect of 50 µM of MK-571 was equivalent to the effect of 1 µM of telaprevir (Figure 1). In the presence of telaprevir, MK-571 increased by approximately 3-fold the anti-HCV effect of telaprevir (Figure 1).

MK-571 blocks HCV replication in a dose-dependent manner

In Huh7.5 cells stably harboring a genotype 1b HCV-SGR, 48 hours of treatment with MK-571 induced a dose-dependent decrease of HCV-SGR RNA, with an EC$_{50}$ of 9.0±0.3 µM (Figure 2A) and a maximal HCV-SGR RNA reduction of approximately 11-fold at 50 µM (Figure 2B). Huh7.5 cell viability was measured and the MK-571 CC$_{50}$ was >100 µM, the highest concentration tested (Table 1).

We next examined HCV-SGR clearance during 2 weeks of treatment with MK-571. Huh7.5 cells harboring an HCV-SGR replicon containing the neomycin resistance gene were grown in the presence of increasing concentrations of MK-571 under G418 selective pressure; under such pressure, only cells efficiently replicating the HCV-SGR...
were able to grow. As shown in Figure 2C, cell growth was not significantly affected by 100 µM of MK-571 in the absence of G418. In contrast, in the presence of G418, MK-571 inhibited cell growth in a dose-dependent manner, as a result of its inhibition of HCV-SGR replication (Figure 2C). These results indicate that MK-571 blocks an intracellular step of the HCV lifecycle.

The anti-HCV activity of MK-571 was then confirmed in a chimeric genotype 2a/2a HCV full-length infectious model (HCV-J6/JFH1) containing a luciferase reporter gene. Huh7.5 cells were infected with HCV-J6/JFH1 in the presence of an increasing concentration of MK-571 and luciferase activity was quantified 48 hours after infection. As with HCV-SGR, MK-571 induced a dose-dependent decrease of HCV-J6/JFH1 RNA replication. However, MK-571 anti-HCV activity appeared higher in the genotype 1b HCV-SGR than the genotype 2a HCV full-length infectious model (Figure 2D).

MK-571 anti-HCV activity is related to its CysLTR1 antagonist property rather than MRP-1 inhibition.

Both MRP-1 and the CysLTR1 receptor are known targets of MK-571. To identify which of them is associated with MK-571 anti-HCV activity, we first measured the anti-HCV effect of two other MRP-1 inhibitors, including probenecid (1 mM) (28) and apigenin homodimer (APN, 0.1 µM) (29). As shown in Figure 3A, none of these two compounds reduced HCV-SGR RNA levels. Furthermore, the addition of 1 mM of probenecid to MK-571 did not modify its anti-HCV activity (data not shown). Together, these results suggest that MRP-1 is not the MK-571 target associated with anti-HCV activity.
Then, the antiviral effect of CysLTR1 natural agonist LTD4 on HCV-SGR was assessed. LTD4 alone did not exert any effect on HCV replication (Figure 3B). However, increasing concentrations of LTD4 reversed the antiviral effect of 50 µM of MK-571 in a dose-dependent manner, with complete reversion achieved at 1 µM (Figure 3C).

We examined whether leukotriene biosynthesis is necessary for the HCV life cycle. The antiviral effect of two potent inhibitors of the 5-lipoxygenase (5-LO), MK-886 and zileuton, was assessed. None of them affected HCV-SGR replication (Figures 3D and 3E), suggesting that leukotriene biosynthesis is not required for HCV replication.

Together these results indicate that the anti-HCV activity of MK-571 is related to the CysLTR1 and that it can be reverted by the addition of the CysLTR1 natural ligand.

Anti-HCV activity of CysLTR1 antagonists zafirlukast, cinalukast and SR2640 and their interaction with MK-571 antiviral activity

The effect of increasing concentrations of CysLTR1 antagonists zafirlukast, cinalukast and SR2640 was tested in the absence or in the presence of 50 µM MK-571 in Huh7.5 HCV-SGR cells. As shown in Figures 4A and 4B, respectively, zafirlukast and cinalukast alone had a modest proviral effect on HCV RNA replication. SR2640 alone increased HCV-SGR RNA levels in a dose-dependent manner, with a maximum increase of 11-fold achieved at a concentration of 20 µM (Figure 4C).

Both zafirlukast and cinalukast reversed the anti-HCV activity of MK-571 in a dose-dependent manner, with full reversion achieved at the highest dose tested (Figures 4D, G and 4E, H). The addition of SR2640 to MK-571 not only reversed its anti-HCV activity, but also increased HCV replication at the highest concentrations tested.
Together, these results suggest that CysLTR1 regulates HCV replication, in a positive or negative way depending on the CysLTR1 antagonist used.

**DISCUSSION**

The approval of highly efficient, well-tolerated DAAs has profoundly changed the HCV treatment landscape. HCV infection can now be cured in most DAA-treated patients within 8 to 12 weeks (4). Despite this unprecedented therapeutic revolution, HCV remains an interesting model to better understand RNA virus life cycles and their interactions with infected hosts.

While studying the modulatory effect of ABC transporter inhibition on the anti-HCV activity of first-generation HCV protease inhibitors, we fortuitously observed an inhibitory effect of MK-571 on HCV replication in a hepatoma cell line harboring an HCV replicon. We further characterized the anti-HCV activity of MK-571 in Huh7.5 cells stably harboring a genotype 1b subgenomic replicon and observed a dose-dependent effect with an EC50 of 9.0 ± 0.3 µM and a maximum HCV RNA level reduction of approximately 11-fold at a concentration of 50 µM of MK-571. Extended treatment with 100 µM of MK-571 cured almost all Huh7.5-SGR-containing cells from their replicons. These results suggest that MK-571 inhibits HCV RNA replication. Although MK-571 antiviral effect was relatively modest, it points out a new, thus far unknown mechanism involving an interaction between HCV and a host cellular pathway that influences its replication.

The lack of anti-HCV activity of APN and probenecid, two specific MRP-1 inhibitors, and their lack of effect on MK-571 antiviral activity suggested that another target of MK-571 is involved in the interaction with the HCV life cycle. CysLTR1 has been described as another binding target for MK-571 (18). CysLTR1 belongs to the G protein-
coupled receptor (GPCR) family. Its activation is linked to the metabolism of phosphatidylinositol and intracellular calcium mobilization (26). CysLTR1 has been shown to activate MAP kinases, to induce cellular differentiation and proliferation, chemotaxis and actin reorganization, to release mediators of inflammation and to regulate hematopoietic stem cells (25). CysLTR1 has been implicated in a number of inflammatory diseases, including asthma and allergic rhinitis (21, 23). Several CysLTR1 receptor-specific antagonists, including montelukast, cinalukast, and zafirlukast have been developed and are used in clinical practice for the treatment of these diseases (30). Recent studies suggested that CysLTR1 is also involved in various types of liver diseases (31). The human CysLTR1 receptor gene encodes a 337 amino acid protein with a calculated molecular mass of 38 kDa, reported to migrate at a molecular weight of approximately 44 kDa in its monomeric form, which contains a nuclear localization signal at its C-terminal end (25).

Our results demonstrate that the antiviral activity of MK-571 is related to the CysLTR1. Indeed, LTD4, the natural agonist of CysLTR1 reversed the anti-HCV effect of MK-571 in a dose-dependent manner, suggesting that MK-571 displacement from CysLTR1 is sufficient to lose anti-HCV activity. Unlike MK-571, LTD4 and 5-LO inhibitors alone had no effect on HCV replication, suggesting that the presence of CysLTR1 rather than its ligand-induced activation is exploited by HCV for its replication. Interestingly, none of the other CysLTR1 antagonists tested, including zafirlukast, cinalukast and SR2640, reduced HCV RNA replication; however, all of them reversed the anti-HCV activity of MK-571. SR2640 induced HCV replication in a dose-dependent manner, suggesting that CysLTR1 has an effect on the HCV life cycle that is not univocal, according to the compound and its binding mode. This differential effect could be
explained by the fact that receptor binders may act as partial agonists, neutral agonists or inverse agonists (32, 33), thereby inducing different biological responses.

In summary, we serendipitously identified MK-571 as an inhibitor of HCV replication in hepatoma cell lines harboring an HCV replicon. MK-571 anti-HCV activity was not related to its well-known effect as an MRP-1 inhibitor, but rather to its CysLTR1 antagonist property. Our results highlight, for the first time, the implication of the CysLTR1 receptor in the HCV life cycle. Overall, our study demonstrates that CysLTR1 is involved in HCV replication, with an impact on both the HCV life cycle and infected cell biology.

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**FIGURE LEGENDS**

**Figure 1.** HCV-SGR RNA levels in Huh7.5-SGR cells quantified by means of RT-qPCR in the absence (black bars) or in the presence (gray bars) of 1 µM telaprevir, without (VEH: vehicle) or in combination with 50 µM of MK-571. The data are shown as mean ± SD of at least three independent experiments. NS: not significant; *p<0.05 and **p<0.01.

**Figure 2.** Anti-HCV activity of MK-571. (A) Dose-response curve and (B) fold-decrease of MK-571 antiviral activity in Huh7.5 cells stably expressing an HCV genotype 1b subgenomic replicon. Data are shown as means±SD from at least two independent experiments performed in triplicate. (C) Crystal violet staining of Huh7.5-SGR cells after two weeks of treatment with different concentrations of MK-571 in the presence of G418; 100 µM of MK-571 was also tested in the absence of G418 for cell viability (right bottom image).

**Figure 3.** (A) Effect of 50µM of MK-571, 1mM of probenicid, and 0.1 µM of apigenin homodimer (APN) on HCV-SGR RNA levels in Huh7.5-SGR cells. (B) Effect of increasing concentrations of LTD4 on HCV-SGR RNA levels in Huh7.5-SGR cells. (C) Effect of increasing concentrations of LTD4 on the antiviral effect of 50 µM of MK-571 in Huh7.5-SGR cells. (D) Effect of increasing concentrations of MK886 on HCV-SGR RNA levels in Huh7.5-SGR cells. (E) Effect of increasing concentrations of zileuton on HCV-SGR RNA levels in Huh7.5-SGR cells. The data are shown as mean ± SD of at least three independent experiments; NS: not significant; *p<0.05.
Figure 4. Effect of zafirlukast (A), cinalukast (B) and SR2640 (C) on HCV-SGR RNA expression in Huh7.5-SGR cells in the absence of MK-571. Effect of zafirlukast (D), cinalukast (E) and SR2640 (F) on HCV-SGR RNA expression in Huh7.5-SGR cells in the presence of 50 µM of MK-571.

Table 1: *In vitro* cellular toxicities of the indicated compound in Huh7.5 cells

| Compound    | CC₅₀ (µM) Huh7.5 cells |
|-------------|------------------------|
| MK-571      | 147.8 ± 6.8            |
| Cinalukast  | > 100                  |
| SR2640      | 33.4 ± 3.9             |
| Zafirlukast | > 100                  |
| Telaprevir  | > 20                   |
Figure 1

HCV-SGR RNA (fold-decrease)

| 1 | 5 | 9 | 13 | 17 | 21 | 25 | 29 | 33 | 37 |
|---|---|---|----|----|----|----|----|----|----|
| * | * | ** |    |    |    |    |    |    |    |

- + Telaprevir
- No Telaprevir

VEH
MK571

Figure 1
Figure 2

A

HCV-SGR replication (% of untreated control)

Concentration of MK-571 (µM)

Log₁₀ concentration of MK-571 (µM)

B

Concentration of MK-571 (µM)

HCV-SGR RNA (fold-decrease)

C

Concentration of MK-571 (µM)

HCV-J6/JFH1 replication (% as untreated control)

D

Log₁₀ Concentration of MK-571 (µM)
Figure 3

A

Concentration of LTD4 (nM)

HCV-SGR RNA (fold-decrease)

B

Concentration of LTD4 (nM)

HCV-SGR replication (percent of untreated controls)

C

Concentration of LTD4 (nM)

HCV-SGR RNA (fold-decrease)

D

Concentration of MK886 (µM)

HCV-SGR replication (percent of untreated controls)

E

Concentration of zileuton (µM)

HCV-SGR replication (percent of untreated controls)
Figure 4

A

B

C

No MK-571

Concentration of zafirlukast (µM)

Concentration of cinalukast (µM)

Concentration of SR2640 (µM)
Figure 4

**D**

HCV-SGR RNA (fold change) vs. Concentration of zafirlukast (µM)

**E**

HCV-SGR RNA (fold change) vs. Concentration of cinalukast (µM)

**F**

HCV-SGR RNA (fold change) vs. Concentration of SR2640 (µM)

+ MK-571 (10 µM)
Figure 4

**G**

Concentration of zafirlukast (µM)

HCV-NSG RNA (fold-decrease)

| Concentration | RNA (fold-decrease) |
|---------------|---------------------|
| 0             | 1                   |
| 10            | 3                   |
| 20            | 5                   |
| 50            | 7                   |
| 100           | 9                   |

+ MK-571 (50 µM)

**H**

Concentration of cinalukast (µM)

HCV-NSG RNA (fold-decrease)

| Concentration | RNA (fold-decrease) |
|---------------|---------------------|
| 0             | 1                   |
| 10            | 3                   |
| 20            | 5                   |
| 50            | 7                   |
| 100           | 9                   |

+ MK-571 (50 µM)

**I**

Concentration of SR2640 (µM)

HCV-NSG RNA (fold change)

| Concentration | RNA (fold change) |
|---------------|-------------------|
| 0             | 0                 |
| 5             | 5                 |
| 15            | 15                |
| 30            | 17                |

+ MK-571 (50 µM)