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Specific inhibition of hepatitis C virus replication by cyclosporin A

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Abstract

The difficulty in eradicating hepatitis C virus (HCV) infection is attributable to the limited treatment options against the virus. Recently, cyclosporin A (CsA), a widely used immunosuppressive drug, has been reported to be effective against HCV infection [J. Gastroenterol. 38 (2003) 567], although little is understood about the mechanism of its action against HCV. In this study, we investigated the anti-viral effects of CsA using an HCV replicon system. Human hepatoma Huh7 cells were transfected with an HCV replicon expressing a chimeric gene encoding a luciferase reporter and neomycin phosphotransferase (Huh7/Rep-Feo). Treatment of the Huh7/Rep-Feo cells with CsA resulted in suppression of the replication of the HCV replicon in a dose-dependent manner, with an IC50 of ~0.5 μg/ml. There were no changes in the rate of cell growth or viability, suggesting that the effect of CsA against HCV is specific and not due to cytotoxicity. In contrast, FK506, another immunosuppressive drug, did not suppress HCV replication. CsA did not activate interferon-stimulated gene responses, suggesting that its action is independent of that of interferon. In conclusion, CsA inhibits HCV replication in vitro specifically at clinical concentrations. Further defining its mode of action against HCV replication potentially may be important for identifying novel molecular targets to treat HCV infection.

Keywords: Hepatitis C virus; Replicon; Cyclosporin A; FK506; ISRE; Cyclophilins

Hepatitis C virus (HCV), which infects 170 million people worldwide, is characterized by chronic liver inflammation and liver fibrogenesis, leading to end-stage liver failure and hepatocellular malignancy [1,2]. Attempts to control HCV infection have been unsatisfactory because of the limited treatment options against the virus. Present therapies against HCV infection are based on high dose administration of interferon (IFN)-α in combination with ribavirin, a synthetic guanosine analog [3,4]. However, success rates remain at around 30–40% of patients treated. Furthermore, treatment with IFN and ribavirin carries a significant risk of serious side effects. Thus, the development of new therapeutic agents is a high priority goal.

Recently, CsA, the most widely used immunosuppressive drug, was reported to be clinically effective against HCV infection [5]. A subsequent controlled trial showed that a combination of CsA with IFN is more effective than IFN monotherapy, especially in patients with a high viral load [6]. In T cells, which are the major cellular targets of CsA, CsA binds to soluble cytosolic proteins called cyclophilins, and the cyclophilin–CsA complexes block calcineurin, which inhibits stimulation of the NFAT-induced genes which are essential for the activation of T cells [7]. However, despite the clinical effectiveness of CsA, little is understood about its anti-viral mechanisms in patients with chronic hepatitis C. In particular, elucidation of the mechanism of the anti-viral effects of CsA may give new insights into the replication of HCV and elucidate potential targets for anti-HCV therapy.

In the present study, we investigated the effects of CsA on the intracellular replication of the HCV genome in vitro, using an HCV replicon system, reported

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recently in a cultured human hepatoma Huh7 cell line [8]. We demonstrated that CsA inhibits HCV replication in vitro substantially and specifically, and that the mechanism of action is independent of that of IFN.

Materials and methods

Drugs. CsA was purchased from Sigma (St. Louis, MO). FK506 was from Alexis Biochemicals (Lausen, Switzerland). Recombinant human IFNα-2b was obtained from Schering-Plough (Kenilworth, NJ).

Cell culture. A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum at 37 °C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in medium containing 200 μg/ml G418 (Wako, Osaka, Japan).

HCV replicon constructs. An HCV subgenomic replicon plasmid, pHCVIbneo-delS, was derived from an infectious HCV clone, HCV-N, genotype Ib [9]. The replicon pHCVIbneo-delS was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotransferase, as described elsewhere (pRep-Feo) [10]. RNA was synthesized from pHCVIbneo-delS and pRep-Feo using T7-polymerase (Lumat LB9501; Promega) and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established (Huh7/Rep-Neo and Huh7/Rep-Feo, respectively).

Reporter constructs. A plasmid, pISRE-TA-Luc (Invitrogen, Carlsbad, CA), expressed luciferase reporter gene under control of the interferon stimulation response element (ISRE). pRL-CMV (Promega), which expresses *Renilla* luciferase gene under the control of the cytomegalovirus early promoter/enhancer, was used as a control for transfection efficiency of pISRE-TA-Luc. A plasmid, pECMV/IRES-Rluc, was used as a control to analyze translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (ECMV-IRES) which mediates translation of HCV non-structure gene of replicon constructs, pHCVIbneo-delS and pRep-Feo. The pECMV/IRES-Rluc expressed mRNA consisted of ECMV-IRES and *Renilla* luciferase reporter gene under control of cytomegalovirus early promoter/enhancer.

Luciferase assays. Luciferase activities were quantified using a luminometer (Promega) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls.

Northern blotting. Total cellular RNA was extracted from cells using ISOGEN (Wako). The RNA was separated by denaturing agarose–formaldehyde–gel electrophoresis and transferred to a Hybond-N^+ nylon membrane (Amersham–Pharmacia Biotech). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for β-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Mannheim, Germany) and visualized using a Fluoro-Imager (Roche).

Western blotting. Ten micrograms of total cell lysate was separated using NuPAGE 4–12% Bis–Tris gels (Invitrogen) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with a monoclonal anti-NS5A antibody (BioDesign, Saco, ME) and detection was carried out in a chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche).

MTS assays. To evaluate cytotoxicity, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

Statistical analyses. Statistical analyses were performed using Student's t test; p values less than 0.05 were considered as statistically significant.

Results

Suppression of hepatitis C virus replication by cyclosporin A (Figs. 1 and 2)

To assess the effects of CsA on the intracellular replication of the HCV genome, Huh7/Rep-Feo cells were cultured with various concentrations of CsA in the medium. The luciferase activities of the Huh7/Rep-Feo

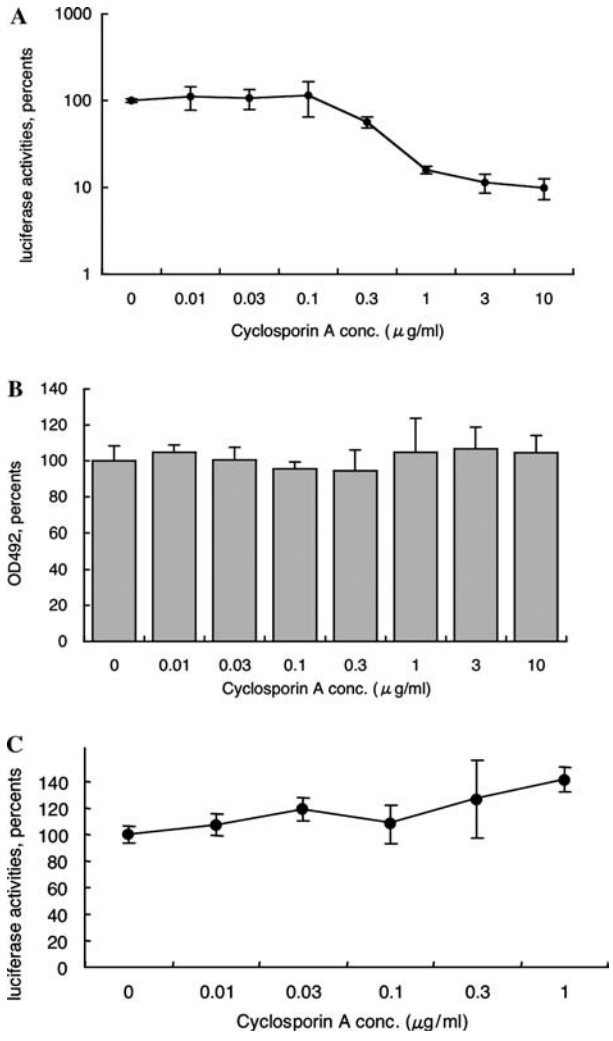

Fig. 1. Suppression of hepatitis C virus replication by cyclosporin A. (A) Huh7/Rep-Feo cells, which constitutively express a hepatitis C virus replicon, enable the quantification of replication levels through the measurement of luciferase activity. Relative log (dose)–response curves in the presence of various concentrations of CsA. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. (B) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of CsA indicated. (C) A plasmid, pECMV/IRES-Rluc, was used to analyze the translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (ECMV-IRES) which mediates translation of HCV non-structure gene of replicon constructs. The pECMV/IRES-Rluc was transfected into Huh7 cells. The transfected cells were cultured in the presence of indicated concentrations of CsA and luciferase activities were measured at 48 h of transfection. The assays were done in triplicate. Error bars indicate means ± 2SD.

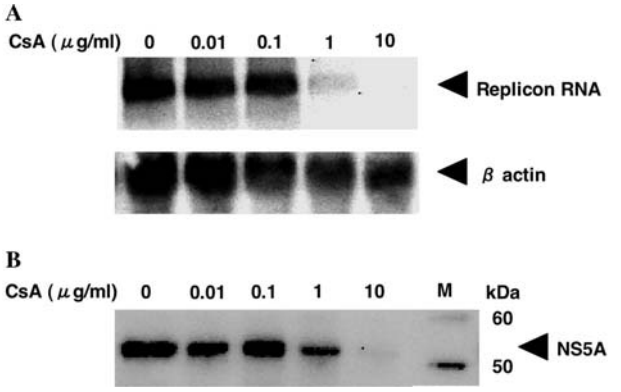

Fig. 2. Suppression of replicon RNA and NS 5A synthesis by CsA. (A) Northern hybridization. The replicon cells described by Seeger were cultured in the presence of the amounts of CsA indicated and harvested at 48 h after exposure. Ten micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the HCV replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with a β-actin probe. Lane 1, Naïve replicon; lane 2, 0.01 μg/ml CsA; lane 3, 0.1 μg/ml CsA; lane 4, 1 μg/ml CsA; and lane 5, 10 μg/ml CsA. (B) Western blotting. Ten micrograms of total cellular protein was electrophoresed in each lane. Monoclonal anti-NS5A antibody and polyclonal anti-luciferase antibody were used as the primary antibodies. Lane 1, sample without CsA; lanes 2–5, cells cultured with CsA at concentrations of 0.01 (2), 0.1 (3), 1 (4), and 10 μg/ml (5). Lane 5, protein size marker, MagicMark (Invitrogen).

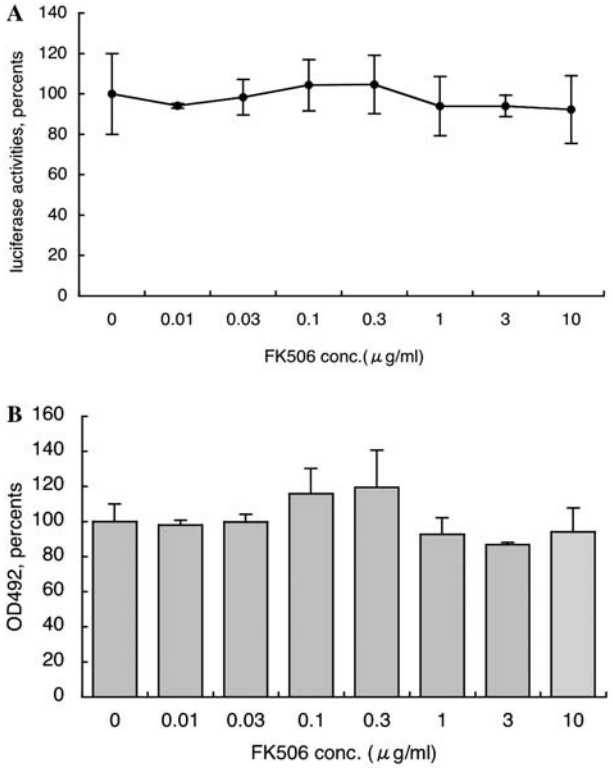

Fig. 3. Absence of an inhibitory effect of FK506. (A) To assess the effects of another immunosuppressive agent, FK506, Huh7/Rep-Feo cells were cultured with various concentrations of FK506 in the medium, and luciferase assays were performed after 48 h of culture. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. (B) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of FK506 indicated.

cells showed that the replication of the HCV replicon was suppressed by CsA in a dose-dependent manner. The luciferase activities were 56% and 16% of the control at CsA concentrations of 0.3 and 1 μg/ml, respectively (Fig. 1A). The MTS assay did not show any effect on cell viablilty or replication (Fig. 1B). Moreover, efficiency of the EMCV-IRES-mediated translation was not affected by CsA (Fig. 1C). These results suggest that the decrease in HCV replication is due to a specific suppressive effect of CsA on HCV replication, and not due to cytotoxicity of CsA or an artificial effect on the EMCV-IRES which direct translation of HCV non-structure protein of the replicon.

In Northern blot analysis (Fig. 2A), levels of the replicon RNA, which were detectable in CsA-negative control cells, decreased substantially following treatment with CsA at concentrations of 1 and 10 μg/ml. Densitometric analysis of the replicon RNA showed that the intracellular levels of the replicon RNA in Huh7/Rep-Feo correlated well with the luciferase activities (data not shown). Similarly, in Western blotting (Fig. 2B), the HCV non-structural protein, NS5A, translated from the HCV replicon, decreased by corresponding amounts in response to treatment with CsA.

Absence of an inhibitory effect of FK506 (Fig. 3)

FK506 (tacrolimus), another immunosuppressive agent, shares many mechanisms of action with CsA. FK506 is another inhibitor for the calcineurin/NFAT pathway and also blocks the activation of the JNK and p38 pathways in T cells [11]. To assess the potential effect of FK506, Huh7/Rep-Feo cells were cultured with FK506 at 0.01–10 μg/ml. The luciferase activities of the cells did not show a significant effect of FK506 on HCV replication at concentrations covering the range achievable clinically.

Cyclosporin A does not elicit an interferon-stimulated response (Fig. 4)

It has been reported that the HCV replicon is highly sensitive to IFN [12]. To determine whether the action of CsA on HCV subgenomic replication involves activation of IFN-stimulated gene responses, the ISRE-luciferase plasmids were transfected into Huh7/Rep-Feo cells and cultured in the presence of CsA and FK506 at concentrations of 0, 0.1, 1, and 10 μg/ml. As a positive control for the activation of ISRE reporter activity, the ISRE-luciferase-transfected cells were cultured with IFNα-2b at concentrations of 0, 0.1, 1, and 10 U/ml. The luciferase activities at 48 h after transfection showed that there were no significant effects of CsA and FK506 on

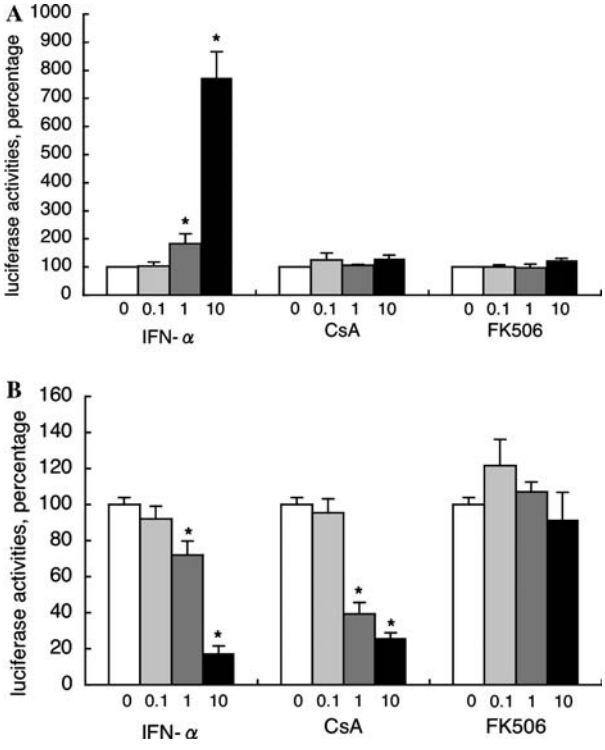

Fig. 4. Cyclosporin A does not elicit an interferon response. (A) ISRE-responsive luciferase reporter plasmids were transfected into cells containing replicon in the presence of the indicated concentrations of IFN (U/ml), CsA, and FK506 (μg/ml) in the culture medium. Luciferase assays were carried out 48 h after transfection. Values are presented as percentages of drug-negative controls. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD. *p value <0.05. (B) Huh7/Rep-Feo cells were cultured with the indicated concentrations of IFN (U/ml), CsA, and FK506 (μg/ml). Luciferase activities of the cell lysates were carried out after 24 h of exposure. Luciferase assays were performed in triplicate. Error bars indicate means ± 2SD.

ISRE-promoter activities, while IFNα-2b had significant effects (Fig. 4A). As reported above, the luciferase activities of the Huh7/Rep-Feo cells showed that the replication of the HCV replicon was suppressed by IFNα-2b, as well as CsA, in a dose-dependent manner, but not by FK506 in this condition (Fig. 4B). These results suggest that the action of CsA on the intracellular replication of HCV replicon is independent of the IFN pathway.

Discussion

Our present results demonstrate that CsA inhibits the intracellular replication of an HCV subgenomic replicon at clinically achievable drug concentrations. The Northern and Western blot analyses revealed that both RNA synthesis and its translation were reduced by CsA in a dose-dependent manner. Treatment of Huh7 cells with CsA did not activate IFN-stimulated gene responses, suggesting that the mechanisms of action of CsA are independent of those of IFN. In addition, FK506, another drug used frequently and which shares an immunosuppressive mechanism with CsA, did not show any inhibitory effect on HCV replication, suggesting that the anti-HCV effect of CsA is not associated with immunosuppressive activity.

CsA and FK506, although structurally dissimilar, have a similar mode of immunosuppressive action in preventing induction of inflammatory gene expression in activated T cells. Both CsA and FK506 bind specifically to a family of soluble cytosolic proteins called immunophilins [7]. CsA is bound by cyclophilins and FK506 is bound by the FK506 binding proteins (FKBPs). The cyclophilin–CsA and FKBPs–FK506 complexes inhibit the phosphatase activity of calcineurin, that mediates phosphorylation, and nuclear translocation of the transcription factor, NFAT, critical in the expression of cytokines and their receptors, respectively [7,13,14]. In our present study, contrary to the effect of CsA on HCV replication, FK506, which has an immunosuppressive activity 100-fold greater than that of CsA [15,16], did not show an inhibitory effect on HCV replication. The findings demonstrate that the anti-viral action of CsA against HCV is not through suppression of NFAT-regulated gene responses but through distinct mechanisms that are not shared with FK506.

Another function of cyclophilins and FKBPs, which CsA and FK506, respectively, are able to block, is a peptidyl–prolyl *cis–trans* isomerase (PPIase) activity. PPIase catalyzes the *cis–trans* conversion of imide peptide preceding prolines [17], and acts as a molecular chaperone, accelerating the slow steps of correct post-translational folding of some proteins [18]. Cyclophilins are present in every compartment of the cell, including the cytoplasm, endoplasmic reticulum (ER), and nucleus [19]. The maturation steps of HCV proteins include processing of the polyprotein by an ER-membrane-bound signal peptidase and by two viral serine proteases [20]. Recent studies have demonstrated the localization of viral non-structural proteins in the ER forming microscopic intracellular structures, called "membranous webs," which is characterized by convoluted ER membranes [21]. Moreover, folding and assembly of HCV structural and non-structural proteins require interaction with ER chaperone proteins such as calreticulin, BiP, and HSP90 [22,23]. Collectively, it is speculated that certain chaperone activities, such as those of cyclophilins, may be crucial for the processing and maturation of the viral proteins and for viral replication. Thus, one most likely mechanism of action against HCV is blocking of the PPIase activities of cyclophilins. The striking difference between the significant effects observed with CsA and the lack of effect of FK506 may be explained by the different enzymatic properties of cyclophilins and FKBPs [24]. Cyclophilins are non-specific PPIases and are able to isomerize all X-Pro bonds.

On the other hand, FKBPs catalyze the isomerization of a limited set of X-Pro bonds. Therefore, inhibition of FKBPs can be overridden by the action of cyclophilins, whereas inhibition of cyclophilins cannot be substituted by the limited substrate specificity of FKBPs [25].

In addition to the anti-viral activity of CsA against HCV, confirmed by the present results, CsA has been reported to show anti-viral effects against HIV-1 through blocking the activities of cyclophilin A [19]. Cyclophilin A binds to the viral *gag* protein with high affinity, and CsA competes with the *gag* protein for the same binding site on cyclophilin A [26]. Moreover, cyclophilin A is packaged into HIV-1 virions and catalyzes *cis–trans* isomerization of the viral capsid protein as a molecular chaperone [27]. It has been shown that the anti-HIV action of CsA does correlate not with the immunosuppressive potential of the compounds but with their capacity to bind to cyclophilins [28]. These reports demonstrate that the anti-viral action of CsA against HIV-1 is through inhibition of the PPIase activities of cellular cyclophilins, as against HCV suggested in this study.

End-stage HCV liver cirrhosis is a major indication for liver transplantation, accounting for approximately 50% of cases in the US and in Europe [29]. However, viral recurrence occurs in all recipients and HCV-graft hepatitis develops in one-third [30]. Decompensated graft cirrhosis following re-infection with HCV is the main cause of death post-transplantation [31]. Long-term immunosuppression is essential for patients who have undergone transplantation. The two most frequently used drugs are CsA and FK506 and usage of FK506 has increased from 0% before 1996 to nearly 80% after 1999 [31], because the early safety and efficacy of an FK506 regimen after liver transplantation has been shown in two multicenter trials [32,33]. More recently, it has been reported that FK506-based immunosuppression is preferable to cyclosporin A during the first year following liver transplantation [34]. As for transplantation for HCV cirrhosis, however, recent studies from two institutes in Spain and the USA report that disease progression after transplantation has accelerated in recent years [29,31], although the reasons for the worsening outcome are under question. The anti-viral activity of CsA against HCV, demonstrated in this study, should be taken into account when selecting the immunosuppressive regimen for the optimum outcome of HCV-infected recipients.

The expanding applications of CsA, however, may cause substantial problems. Especially, an undesired immunosuppression that may lead to an immunocompromised status and may interfere with the effects of anti-infectious drugs such as IFN. Some cyclosporine analogs that fail to block T cell activation are still able to inhibit the PPIase activity [16,35]. Some of these non-immunosuppressive cyclosporine analogs were, in fact, equal or even superior in anti-HIV activity to immunosuppressive types [36]. Therefore, one solution to overcome the problem due to immunosuppression would be to consider the use of such cyclosporine analogs.

In conclusion, CsA inhibits HCV replication in vitro substantially and specifically. Considering the limited therapy options against HCV infection and the unsatisfactory therapy outcome, with half of the patients unable to eradicate the virus, CsA potentially becomes available as an effective treatment against HCV infection. Further defining the mechanism of action of CsA against HCV replication potentially may be important for identifying novel molecular targets to terminate HCV infection.

Acknowledgments

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