Antiviral Research 108 (2014) 79–87

Contents lists available at ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Suppression of hepatitis C virus replication by cyclin-dependent kinase inhibitors

Tsubasa Munakata [a,b,*], Makoto Inada [b], Yuko Tokunaga [a], Takaji Wakita [c], Michinori Kohara [a], Akio Nomoto [b,d]

[a] Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan
[b] Department of Microbiology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
[c] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
[d] Institute of Microbial Chemistry, 3-14-23 Kamiohsaki, Shinagawa-ku, Tokyo 141-0021, Japan

ARTICLE INFO

Article history:
Received 5 February 2014
Revised 2 May 2014
Accepted 23 May 2014
Available online 2 June 2014

Keywords:
HCV
CDK inhibitor
Rb
Replicon
Chimera mice

ABSTRACT

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis. Although the standard therapy for HCV-infected patients consists of pegylated interferon plus ribavirin, this treatment is associated with serious side effects and high costs, and fails in some patients infected with specific HCV genotypes. To address this problem, we are developing small-molecule inhibitors of cyclin-dependent kinases (CDKs) as novel anti-HCV drug candidates. Previous data showed that HCV replication is inhibited by retinoblastoma protein, which is itself inactivated by CDK-mediated phosphorylation. Here, we report that CDK inhibitors suppress HCV replication *in vitro* and *in vivo*, and that CDK4 is required for efficient HCV replication. These findings shed light on the development of novel anti-HCV drugs that target host factors.

1. Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, and persistent HCV infection is a major risk factor for the development of a range of liver diseases, including cirrhosis and hepatocellular carcinoma (HCC). As an estimated 170 million people worldwide are infected with HCV, this virus is an increasing concern for global public health (De Francesco and Migliaccio, 2005). HCV is classified into 6 major genotypes (designated 1–6) and several subtypes (designated a, b, c, etc.) (Simmonds et al., 2005). Types 1a and 1b are the most common, accounting for about 60% of global infections. Currently, the standard therapy for HCV-infected patients consists of treatment with pegylated interferon (PEG-IFN) plus ribavirin (Feld and Hoofnagle, 2005). This combination therapy has been quite successful in patients with HCV genotype 2 or 3 infections, leading to sustained virologic response (SVR) in more than 80% of patients treated (Pawlotsky, 2009). However, in patients infected with HCV genotype 1 or 4, only about 50% of treated individuals achieve SVR (Keeffe, 2007). Furthermore, treatment is often associated with serious side effects and high costs.

HCV is an enveloped virus with a positive-strand RNA genome. The 9.6-kb HCV genome encodes a single polyprotein processed by both cellular and viral proteases into at least 10 distinct structural (core, E1, E2, and p7) and nonstructural (2, 3, 4A, 4B, 5A, and 5B) proteins. Among these proteins, essential viral enzymes serve as potential candidates for targeting by antiviral therapy. Specifically, the NS3/4A serine protease and NS5B RNA polymerase have been the most popular targets (Waters and Nelson, 2006). A number of competitive inhibitors of NS3, as well as nucleoside and non-nucleoside inhibitors of NS5B, are being developed, although emergence of resistance to inhibitors has been observed *in vivo* and *in vitro* (Sarrazin et al., 2007). For instance, a single mutation is sufficient to confer resistance to a viral protease inhibitor, BILN 2061 (Lamarre et al., 2003; Lin et al., 2005). Very recently, the standard of care for treating chronic HCV infection has changed to a triple therapy of PEG-IFN, ribavirin and an HCV protease inhibitor such as telaprevir, boceprevir or vaniprevir (Macartney et al., 2014; Manns et al., 2012). However, emergence of resistance-associated variants after failed triple therapy remains a matter of concern, and severe side-effects such as anemia limit the choice of patients (Barnard et al., 2013; Poordad et al., 2013), suggesting that a novel approach is necessary to treat HCV infection.

Host mechanisms that regulate cell-cycle progression and cellular proliferation are disrupted in HCC associated with HCV

* Corresponding author at: Department of Microbiology and Cell Biology, Tokyo Metropolitan Institute of Medical Science, 2-1-6 Kamikitazawa, Setagaya-ku, Tokyo 156-8506, Japan. Tel.: +81 3 5316 3122; fax: +81 3 5316 3137.
E-mail address: munakata-tb@igakuken.or.jp (T. Munakata).

http://dx.doi.org/10.1016/j.antiviral.2014.05.011
0166-3542/

(Mayhew et al., 2007; Reed et al., 2010). Among cell-cycle regulators, retinoblastoma protein (Rb) plays a major role in controlling the G1- to S-phase transition and mitotic checkpoints through a repressive effect on E2F transcription factors (Chellappan et al., 1991; Chen et al., 2009). Rb functions as a tumor suppressor, and is targeted by oncoproteins expressed by DNA tumor viruses (Khidr and Chen, 2006; Knudson, 1971). In previous studies, we found that the NS5B polymerase forms a complex with Rb, targeting Rb for degradation (Munakata et al., 2007, 2005). This interaction leads to the activation of E2F-responsive promoters, and promotes progression of the cell cycle from G1- to S-phase in cells expressing NS5B. This interaction also inhibits NS5B polymerase activity, as Rb binds near the NS5B polymerase catalytic site. Given that the S phase facilitates HCV replication and translation, Rb thus is a negative regulator of HCV replication by two mechanisms (Honda et al., 2000; Scholle et al., 2004).

In addition to degradation, Rb can be inactivated by phosphorylation (Buchkovich et al., 1989). Cyclin-dependent kinases (CDKs) phosphorylate Rb at multiple sites to suppress the repressive function of Rb (Knudsen and Wang, 1996). In quiescent cells or cells in early G1, Rb binds to the E2F transcription factors and blocks the E2F transactivation domains, recruiting transcriptional co-repressors and resulting in the repression of E2F-responsive promoters (Chen et al., 2009). Under normal conditions, initiation or progression through the cell cycle requires growth signals that lead to the sequential activation of the CDK-cyclin complexes CDK4/6-cyclin D and CDK2-cyclin E, leading in turn to hyperphosphorylation of Rb and the release of active E2F (Simanis and Nurse, 1986; Uhlmann et al., 2011). In the present study, we used small-molecule CDK inhibitors to activate Rb (Krystof and Uldrijan, 2010; Whittaker et al., 2004). We found that CDK inhibitors suppress HCV replication *in vitro* and *in vivo*, and that CDK4 is required for efficient HCV replication. These findings shed light on the development of novel anti-HCV drugs that target host factors.

2. Materials and methods

2.1. Cells

Human hepatoma cells HuH-7 and its derivatives, including Huh7.5.1, were grown in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA; GlutaMax grade) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin, at 37 °C in a humidified atmosphere with 5% CO_2. The 2–3 cells were a HuH-7-derived cell line containing autonomously replicating, genome-length, dicistronic, selectable HCV RNAs that were derived from the genotype-1b HCV-N strain; the 2–3 cell line was cultured as above, with addition of 300 μg/ml G418 (Ikeda et al., 2002). Y19 and R6FLR-N were HuH-7-derived cell lines containing autonomously replicating, subgenomic, dicistronic, selectable HCV RNAs derived from genotype-2a JFH-1 and genotype-1b HCR6 and HCV-N strains, respectively; these cell lines were cultured as above, with the addition of 500 μg/ml G418. The cognate cell lines, interferon-cured progeny cell lines 2-3c and Y19c, were generated from the respective parent lines and were maintained as described previously (Ikeda et al., 2002), and contained no detectable HCV RNA by RT-PCR assay.

2.2. Viruses

Cell culture-infectious genotype-2a JFH-1 viruses were harvested from the culture medium supernatant of RNA-transfected HuH-7 cells, and stored at −80 °C until use. Huh7.5.1 cells were infected with JFH-1 virus as described previously (Munakata et al., 2007; Wakita et al., 2005), and HCV RNA titers of the infected cells were measured by real-time RT-PCR using a Light Cycler (Roche, Indianapolis, IN). Briefly, one microgram of RNA was reverse-transcribed and amplified using RNA Master Hybridization Probes Kit (Roche) with JFH-1 sense 62–81 (5′-TTC ACG CAG AAA GCG CCT AG-3′) and antisense 311–293 (5′-ACT CGC AAG CGC CCT ATC A-3′) primers, and was detected with LC179-207 (5′-GGA AGA CTG GGT CCT TTC TTG GAT AAA CC-3′-FITC) and LC209-233 (LC-Red-5′-ACT CTA TGC CCG GCC ATT TGG GCG T-3′) probes. Testing revealed that Huh7.5.1 + JFH-1 cells harbored approximately 1×10^7 copies of HCV genome per microgram of RNA.

2.3. CDK inhibitors

Roscovitine, CDK4 inhibitor, CDK2 inhibitor III, fascaplysin, aminopurvalanol A, and indirubin-3′-monoxime-5-sulfonic acid were purchased from Merck Chemicals (Philadelphia, PA); each was prepared as a solution in dimethyl sulfoxide (DMSO). Roscovitine was also purchased from ALEXIS Biochemicals (Farmingdale, NY). Detailed features of each inhibitor including IC50 for a target CDK are shown in Table 1, based on the manufacturer's information (McClue et al., 2002; Meijer et al., 1997).

For treatment of the replicon-carrying and HCV-infected cells, cells were seeded into 6-well plates and grown to 50% confluence. Inhibitors then were added to the culture medium at the indicated concentrations and were incubated for 24–48 h. Cell extracts were then processed to isolate protein (for immunoblots) or total RNA (for real-time RT-PCR).

2.4. Plasmids

CDK4 expression vector, pCMV6XL5-hCDK4, was purchased from Origene. The cDNA encoding cyclin D1 was cloned from a cDNA library of HuH-7 cells, and was subcloned into pcDNA3.1-zeo(+) (Invitrogen) to construct the *cyclin D1* expression vector, pcDNA3.1zeo(+)-hCyclinD1. For over-production of CDK4 or cyclin D1, Huh7.5.1 + JFH-1 cells were seeded into 6-well plates 24 h before transfection and grown to 50% confluence. At the time of transfection, culture medium was replaced with fresh medium without antibiotics. Cells then were transiently transfected with one of the expression plasmids using FuGENE 6 reagents (Roche). Protein extracts were prepared for immunoblots at 48 h after transfection.

2.5. RNA interference

For knockdown of cyclin D1, CDK2, CDK4, CDK6, and MDM2, siGENOME SMART pool siRNAs were purchased from Dharmacon (Pittsburgh, PA). For knockdown of Rb, Stealth RNAi siRNA was purchased from Invitrogen. As negative controls, non-targeting siRNAs and those against green fluorescent protein (GFP) were used. Further analysis of CDK4 knockdown was performed using the four individual siRNA molecules that are normally combined in the CDK4 SMART pool. Transfection with siRNAs was carried out using Lipofectamine 2000 (Invitrogen) as described previously (Munakata et al., 2007).

2.6. Luciferase assays

HCV replicon cells were seeded in 96-well plates at a density of 4×10^3 cells/well. After 24-h incubation, inhibitors were added to the growth medium, and after an additional 72-h incubation, luciferase assays were performed using the Bright-Glo luciferase assay kit (Promega, Madison, WI) according to the manufacturer's instructions. At the same time, cell viability was measured by WST-8 assay (Cell Counting Kit-8; Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Table 1
Inhibitors of cyclin-dependent kinases (CDKs).

Inhibitor	Chemical name	Target CDK (*in vitro* IC50)	Mw
A	Roscovitine	CDK1 (650 nM),CDK2 (100 nM),CDK5 (200 nM)	354.5
B	CDK4 inhibitor	CDK1 (2.1 μM), CDK2 (520 nM), CDK4 (76 nM)	404.2
C	CDK2 inhibitor III	CDK1 (4.2 μM), CDK2 (500 nM), CDK4 (215 μM)	400.5
D	Fascaplysin	CDK4 (350 nM), CDK6 (3.4 μM)	306.8
E	Aminopurvalanol A	CDK1 (33 nM), CDK2 (28 nM), CDK5 (20 nM)	403.9
F	Indirubin-3′-monoxime-5-sulfonic acid	CDK1 (5 nM), CDK5 (7 nM)	357.3

Mw, molecular weight.

2.7. Immunoblots

Cell extracts were prepared in chilled lysis buffer (20 mM Tris–HCl [pH 7.5], 150 mM NaCl, 10 mM Na_2EDTA, 1% [v/v] Nonidet P-40, 10% [v/v] glycerol, and 2 mM dithiothreitol) supplemented with 1 mM phenylmethylsulfonyl fluoride and 2 μg/ml aprotinin. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and subsequent immunoblotting were performed as described previously (Munakata et al., 2005), using mouse monoclonal antibodies against actin (C4; Chemicon, Billerica, MA), CDK4 (DCS-35; Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D1 (DCS-6; Santa Cruz Biotechnology), HCV core (C7-50; Affinity BioReagents, Rockford, IL, USA), HCV NS3 (1828; ViroStat, Portland, ME, USA), HCV NS5A (#950328; Biken, Osaka, Japan), and Rb (G3-245; BD Biosciences, San Diego, CA), and rabbit polyclonal antibodies against BubR1 (A300-995A; Bethyl Laboratories, Montgomery, TX), CDK2 (M2; Santa Cruz Biotechnology), CDK6 (C-21; Santa Cruz Biotechnology), phospho-Rb Ser780 (#9307; Cell Signaling Technology, Danvers, MA) and HCV NS5B (ab35586; Abcam, Cambridge, MA).

2.8. Chimeric mice

Chimeric mice harboring a functional human liver cell xenograft were purchased from PhoenixBio (Hiroshima, Japan). Six weeks after human hepatocyte transplantation, we intravenously injected each mouse with an HCV-infected patient's serum containing 10^6 copies of HCV genotype 1b (HCR6; Accession No. AY045702) (Nakagawa et al., 2007). Protocols for the animal experiments were approved by the Ethics Committee of Tokyo Metropolitan Institute of Medical Science. Animals received humane care according to the guidelines of the National Institutes of Health. The HCV-infected patient who provided the serum provided written informed consent before blood sampling.

The 14-day drug administration was performed as follows. Roscovitine-treated mice received intravenous once-daily (day 0–day 13) injections of 25 or 50 mg/kg roscovitine. IFN-treated mice received subcutaneous twice-weekly (days 0, 3, 7, and 10) injections of 30 μg/kg PEG-IFNα-2a (Chugai, Tokyo, Japan). This IFN dose, administered approximately every 3 days, represented a 10-fold excess when compared to the regimen used once daily in clinical treatment. For all animals (mono- or combined therapy), blood was sampled on days −1, 1, 3, 7, 10, and 14.

For quantification of HCV RNA, total RNA was purified from 1 μl of serum from each mouse by the acid guanidinium–phenol–chloroform method, and HCV RNA was quantified by real-time RT-PCR as described previously (Nakagawa et al., 2007; Takeuchi et al., 1999). For end-point analysis, total RNA was prepared from the liver tissue of chimeric mice, and HCV RNA was quantified as above. RNA analysis of human actin, CDK2, CDK4, CDK6, and MDA5 was performed using TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA). Immunoblots of CDK proteins were performed as described above, using total protein lysates prepared by Tissue Lyser (Qiagen, Valencia, CA).

Human serum albumin in the blood of chimeric mice was measured using the Alb-II kit (Eiken Chemical, Tokyo, Japan) according to the manufacturer's instructions. Serum alanine aminotransferase (ALT) levels were measured using a Transaminase-CII Test A kit (Wako Pure Chemical Industries, Osaka, Japan).

2.9. Statistical analysis

Analyses were performed by two-tailed Student's *t* test using Excel 2003 (Microsoft, Redmond, WA). Mean and standard deviations were derived from at least three separate experiments. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. CDK inhibitors suppress HCV replication

Our preliminary experiments employed three distinct HuH-7-derived human hepatoma cell lines. Cells of this background are known to support the autonomous replication of dicistronic HCV RNA replicons. The first of these cell lines (called R6FLR-N) contained a subgenomic replicon of the HCR6 and HCV-N strain of genotype 1b; this replicon produced only the NS3-NS5B region of the HCV polyprotein (Nakagawa et al., 2007). This cell line also expressed firefly luciferase, which enabled us to quantify replicon activity by measuring luciferase activity. The second cell line (called 2–3) contained replicating genome-length RNA that produced all of the proteins of HCV-N strain (Ikeda et al., 2002). The third cell line (called 2-3c) served as a control, and consisted of a cognate cell line that did not contain any HCV RNA.

In order to determine the effect(s) of CDK inhibition, we initially tested two CDK inhibitors (A and B; Table 1) in these HuH-7-derived cells, and examined HCV replication in the treated cells. As shown in Fig. 1a, CDK inhibitors A and B were able to suppress HCV replication in R6FLR-N replicon cells without affecting cell viability. Moreover, inhibitors A and B reduced the amount of HCV NS5B polymerase as well as that of phosphorylated Rb (Fig. 1b). There were no apparent changes in the amount of total Rb. For both A and B, the 50% effective concentrations (EC_{50}s) for suppression of HCV replication were submicromolar, and other CDK inhibitors, except F, were also able to suppress HCV replication (Supplementary Fig. 1). However, in 2–3 replicon cells, inhibitor B, but not A, suppressed HCV replication, as shown by the immunoblots of HCV NS5A and NS5B proteins (Fig. 1c). Although the effective dose of inhibitor B was higher in 2–3 than in R6FLR-N, this CDK inhibitor suppressed the replication of HCV of genotype 1b *in vitro*.

Next, we examined whether CDK inhibition suppressed the replication of HCV of other genotypes. For this purpose, we used a cell line (called Y19) that contained a subgenomic replicon of the JFH-1 strain of HCV genotype 2a, and a cognate cell line (called Y19c) that did not contain any HCV RNA (Kato et al., 2003), and assessed viral replication by real-time reverse transcription polymerase chain reaction (RT-PCR). Of the six CDK inhibitors tested (A–F; Table 1),

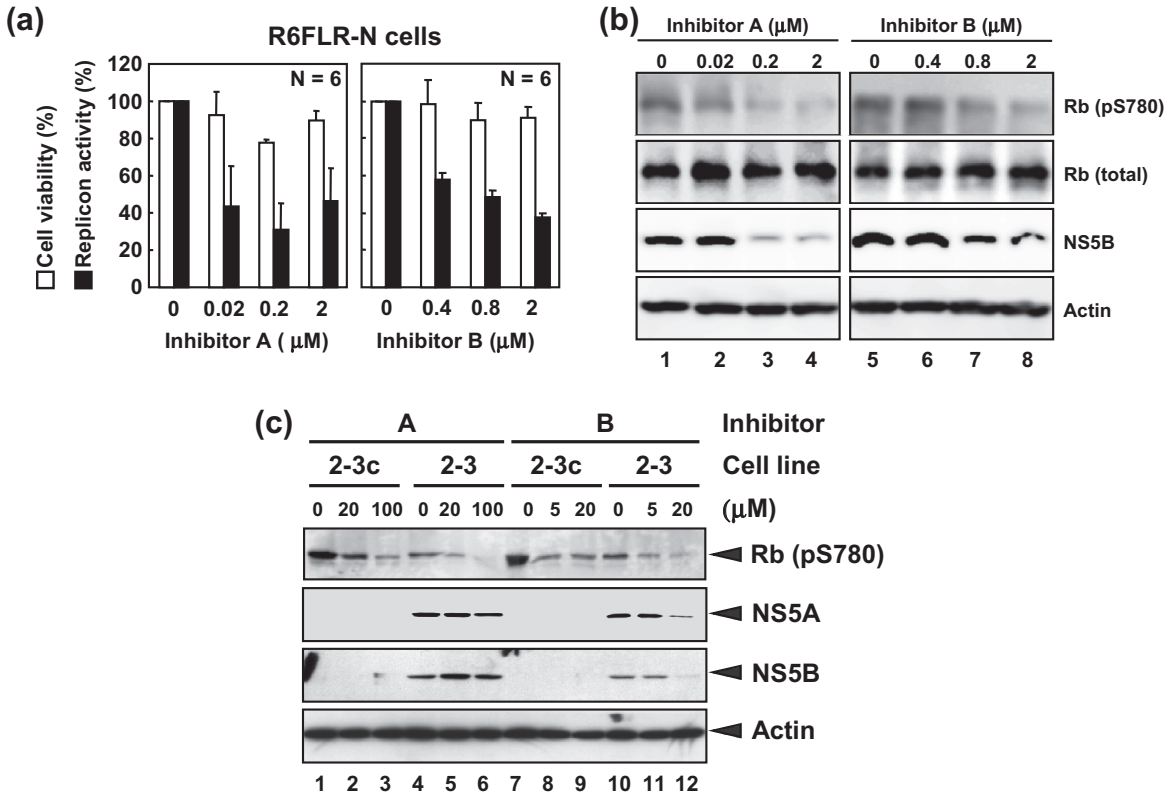

Fig. 1. CDK inhibitors suppress replication of HCV genotype 1b replicon. (a) Inhibition of HCV replicon activity in R6FLR-N cells. After addition of CDK inhibitor, HCV replication was measured by luciferase assay, and cell viability was measured by water-soluble tetrazolium (WST) salts assay. Data represent means ± SD from six experiments. (b) Reduction of HCV NS5B protein in R6FLR-N cells. After addition of CDK inhibitor, total protein was isolated and subjected to immunoblot analysis for HCV NS5B, Rb phosphorylated at Ser780 (pS780; confirmation of CDK inhibition), total Rb, and actin (loading control). CDK inhibitors decreased the amount of phosphorylated Rb in a dose-dependent manner. (c) Inhibition of HCV replicon activity of 2–3 cells. After addition of CDK inhibitor, total protein was isolated and subjected to immunoblot analysis for HCV NS5A, HCV NS5B, Rb phosphorylated at Ser780, and actin. As expected, 2-3c cells did not produce HCV proteins.

five (A, B, C, D, and E) were found to reduce the amount of HCV replicon RNA (Fig. 2a), without affecting cell viability (Supplementary Fig. 1b). We also found that these five inhibitors reduced the amounts of HCV NS5B and phosphorylated Rb proteins, as compared to controls (Fig. 2b). Specifically, inhibitors A, B, and E decreased HCV RNA and protein in a dose-dependent manner. Thus, CDK inhibitors suppressed the replication of genotype-2a HCV *in vitro*.

3.2. CDK inhibitors suppress HCV infection

The preceding experiments demonstrated the effects of CDK inhibitors on HCV replication in three distinct replicon cell lines. To address the effects of CDK inhibition on HCV infection, we employed a model of HCV infection in cultured hepatocytes. Specifically, we used the Huh7.5.1 + JFH-1 cell line, which corresponds to HuH-7-derived cells (designated Huh7.5.1) that have been infected *in vitro* with the JFH-1 strain of HCV (Wakita et al., 2005). HCV infection and reproduction was assayed using RT-PCR detection of HCV RNA genome levels. We found that CDK inhibitors B and E provided dose-dependent reductions in the levels of viral RNA in HCV-infected cells (Fig. 3a), demonstrating the suppression of HCV infection by CDK inhibition. Our data indicated that inhibitor B was the most effective of the 6 inhibitors tested. Inhibitor B was reported to selectively target CDK4 (Table 1) (Matsushime et al., 1992). Therefore, we hypothesized that CDK4 is a positive regulator of HCV replication in infected hepatocytes (Fig. 3b; Section 4).

In order to further demonstrate our hypothesis, we examined whether depletion of Rb, a downstream target of CDK4, rescued suppression of HCV replication by inhibitor B. For this purpose, we first knocked down endogenous Rb using small interfering RNA (siRNA) transfection, and performed HCV replication inhibition assays (Fig. 3c). We found that the inhibitor B-dependent suppression of HCV replication was significantly attenuated by Rb knockdown (Fig. 3d and e). These data clearly indicate that CDK4 affects HCV replication through Rb.

3.3. CDK4 is a host factor required for HCV replication

In order to investigate whether CDK4 is actually required for HCV replication, we first performed RNA interference (RNAi) for knockdown of CDK4 in Y19 replicon cells. Immunoblot analysis demonstrated that knockdown of CDK4 reduced the amounts of CDK4 protein, as well as those of phosphorylated Rb and HCV NS5B proteins, as was the case with CDK inhibitors (Fig. 4a). To further examine whether CDK4 and/or its partner cyclin D are required for the HCV life cycle, we again used RNAi to perform knockdown of CDK4 and cyclin D1 in Huh7.5.1 + JFH-1 cells. Although cyclin D consists of D1, D2, and D3 sub-types, D2 and D3 are not normally produced in the liver, while cyclin D1 is induced in hepatic tumor cells (Musgrove et al., 2011; Teramoto et al., 1999). We therefore focused on cyclin D1 in our analysis. Following transfection with siRNAs that targeted the mRNAs encoding CDK4 or cyclin D1, we used immunoblotting to determine the levels of CDK4, cyclin D1, and HCV core protein. As shown in Fig. 4b, RNAi against CDK4 impaired the production of CDK4 protein and reduced levels of HCV core protein; RNAi against cyclin D1 reduced cyclin D1 production, but did not decrease HCV core protein levels. To demonstrate the specificity of CDK4 knockdown, we transfected

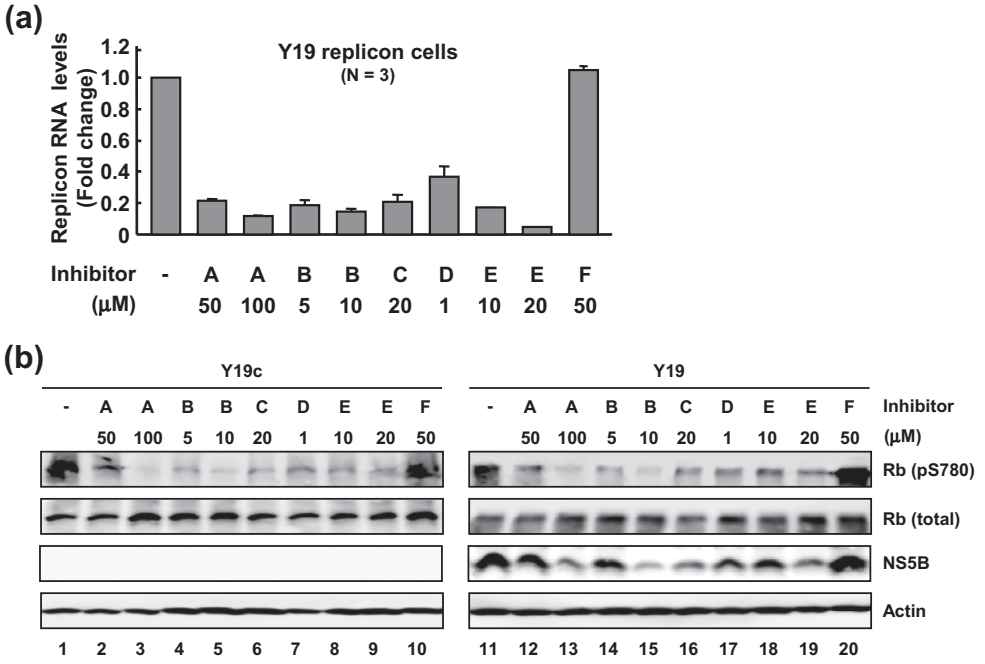

Fig. 2. CDK inhibitors suppress replication of HCV genotype 2a replicon. (a) Inhibition of expression of HCV replicon RNA levels in Y19 cells. After addition of CDK inhibitor, total RNA was isolated, and HCV replicon RNA levels were quantified by real-time RT-PCR. Data represent means ± SD from three experiments. (b) Inhibition of the production of HCV protein levels in Y19 cells. After addition of CDK inhibitor, total protein was isolated and subjected to immunoblot analysis for HCV NS5B, Rb (pS780), total Rb, and actin. CDK inhibitors A–E decreased the amount of phosphorylated Rb and HCV NS5B. As expected, Y19c cells did not express HCV protein.

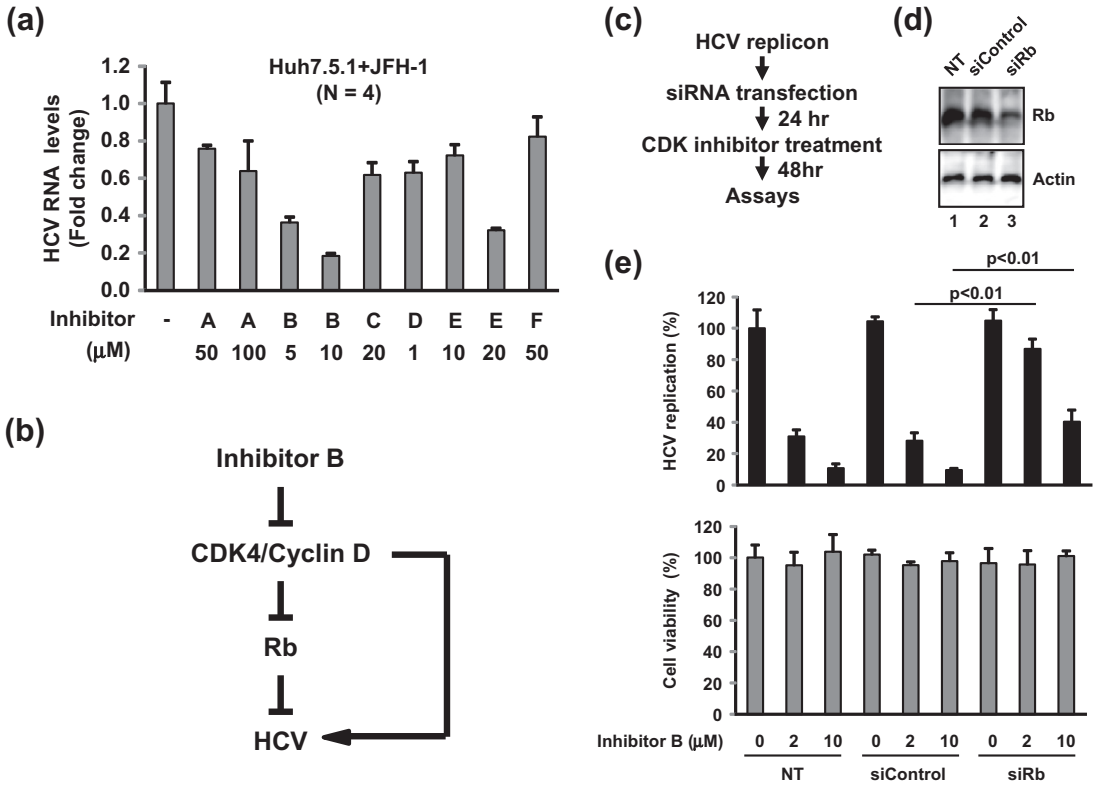

Fig. 3. CDK inhibitors suppress HCV infection in cultured hepatocytes. (a) Inhibition of the expression of HCV genome RNAs in Huh7.5.1 + JFH-1 cells. After addition of CDK inhibitor, total RNA was isolated, and HCV RNA levels were quantified by real-time RT-PCR. Data represent means ± SD from three experiments. (b) Schematic of the roles of CDK inhibitor B in regulation of HCV. Under standard conditions, CDK4 phosphorylates and counteracts Rb, so permitting HCV replication; CDK4 also may play roles independently of Rb. (c) Protocol for rescue assay of CDK inhibitor treatment by Rb knockdown. (d) Immunoblot analysis of endogenous Rb. Knockdown of Rb by RNAi (40 nM) is shown. (e) Attenuation of inhibitor B-dependent suppression of HCV replication. Top panel, HCV replication; bottom panel, cell viability. Decreased HCV replication levels were partially restored by Rb knockdown. Data represent means ± SD from four independent experiments.

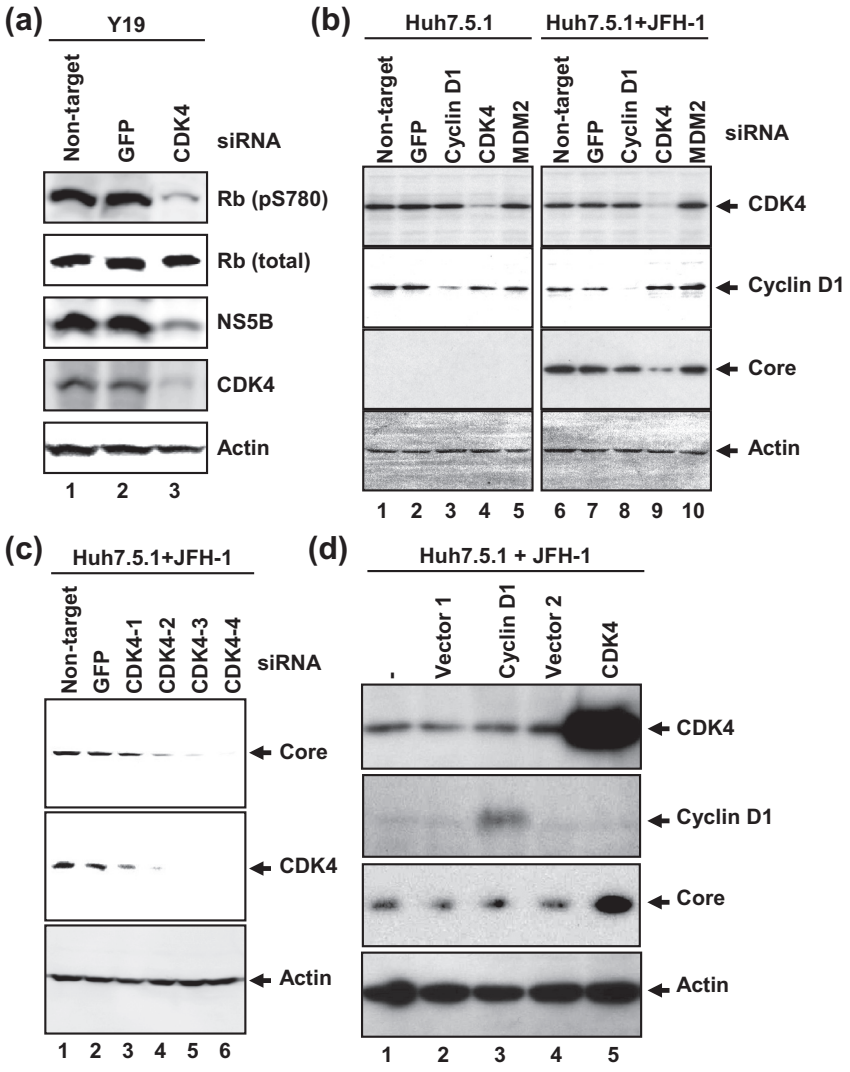

Fig. 4. CDK4 is required for efficient HCV replication. (a) Inhibition of HCV NS5B production by knockdown of CDK4 in HCV replicon cells. In Y19 cells, siRNA-mediated knockdown analysis was performed, and reduction of amounts of CDK4, phosphorylated Rb and HCV NS5B is shown by immunoblot. (b) Inhibition of production of HCV core by knockdown of CDK4, but not by knockdown of cyclin D1. To confirm the validity of inhibitor experiments, siRNA-mediated knockdown analysis was performed in both Huh7.5.1 and Huh7.5.1 + JFH-1 cells. Note that HCV core was not expressed in Huh7.5.1 cells. (c) Inhibition of the production of HCV core by knockdown of CDK4 using four separate siRNA molecules. Among the four, three siRNAs exhibited CDK4 knockdown and down-regulation of HCV core protein abundance. (d) Enhancement of the production of HCV core by over-production of CDK4, but not that of cyclin D1. For all 3 panels, total protein was isolated following siRNA exposure or transfection and subjected to immunoblot analysis for CDK4, cyclin D1, core protein, and actin (loading control).

Huh7.5.1 + JFH-1 cells separately with four distinct siRNAs. We again observed reductions in CDK4 and HCV core abundance in cells transfected with three of these four CDK4-specific siRNAs (CDK4-2, CDK4-3, and CDK4-4) (Fig. 4c). Notably, the siRNA (CDK4-1) that failed to impair HCV core abundance also failed to reduce CDK4 levels, indicating the specific requirement of CDK4 for HCV propagation.

In order to further clarify the role of CDK4 in the HCV life cycle, we overexpressed *CDK4* in Huh7.5.1 + JFH-1 cells, and observed increased levels of both CDK4 and HCV core protein (Fig. 4d). In contrast, overexpression of *cyclin D1* cDNA increased the levels of cyclin D1 protein, but not those of HCV core protein (Fig. 4d). These findings were consistent with the inhibitor assays, and suggested that CDK4 is a host factor required for HCV replication.

CDK4, along with CDK2 and CDK6, is classified as an interphase CDK (Malumbres and Barbacid, 2009). This fact, combined with the results of our CDK inhibitor analysis, suggested that CDK2 and CDK6 might also function as regulators of HCV. Therefore, we repeated our knockdown analysis with siRNAs against the transcripts encoding CDK2 and CDK6. As seen with CDK4, we found that cells with impaired production of CDK2 or CDK6 also exhibited reduced levels of HCV core protein (Fig. 5a and b). Interestingly, knockdown of the interphase CDKs induced overproduction of the mitotic checkpoint protein BubR1 (Fig. 5b), suggesting that normal mitotic progression is required for HCV replication (Elowe, 2011).

3.4. CDK inhibition reduces serum HCV titer in mouse model of HCV infection

All of the above experiments were performed *in vitro*; we next addressed the potential *in vivo* activity of a CDK inhibitor in an animal model of HCV infection. Specifically, we tested the anti-HCV effect of inhibitor A (roscovitine; seliciclib;(McClue and Stuart, 2008; Nutley et al., 2005)) in HCV (genotype 1b)-infected chimeric mice with humanized livers (Nakagawa et al., 2007; Tateno et al., 2004). Although the data presented to date indicate that inhibitor B (CDK4 inhibitor) is the most effective in suppressing HCV

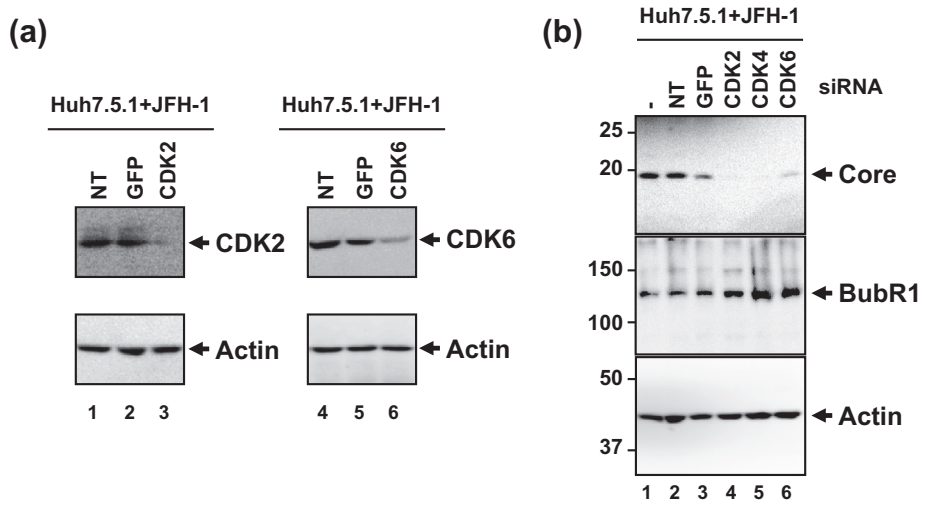

Fig. 5. Interphase CDKs are required for HCV replication. (a) Knockdown of CDK2 or CDK6 in Huh7.5.1 + JFH-1 cells was shown by immunoblot analysis. (b) Expression of HCV core protein was inhibited by knockdown of CDK2, CDK4, and CDK6 in Huh7.5.1 + JFH-1 cells. Immunoblot of BubR1 demonstrated that this cell-cycle checkpoint protein was induced by the reduced levels of each CDK.

replication, it has no valid data on *in vivo* use, which made it difficult to determine the dose for chimeric mice. As shown in Fig. 5, CDK2 was also found to be required for HCV replication, and roscovitine targets CDK2 (Table 1) and has lower EC50 for suppression of HCV replication than inhibitor B in R6FLR-N cells (Supplementary Fig. 1). In addition, this drug is undergoing a phase IIb clinical trial as monotherapy for non-small cell lung carcinoma, and is therefore well characterized, which facilitated our experiment in mice. We administered inhibitor A intravenously over a period of 14 days, both as monotherapy and in combination with subcutaneously-injected polyethylene glycol-conjugated interferon alpha (PEG-IFN). Inhibitor A significantly ($P < 0.05$) reduced serum HCV RNA levels when administered as a 50-mg/kg monotherapy (Fig. 6b), but did not demonstrate efficacy as a 25-mg/kg monotherapy (Fig. 6a). In addition, while treatment with PEG-IFN alone reduced HCV titer by more than one log at day 14, combined administration of PEG-IFN and inhibitor A (at either dose level) provided further reductions in serum HCV RNA levels (Fig. 6a and b). Detailed HCV titers in the serum of each chimeric mouse are shown in Supplementary Fig. 2. Judging from body weight, human albumin levels and ALT levels, our experiments did not significantly affect the status of chimeric mice (Supplementary Fig. 3). We also analyzed the liver tissues from a set of experiments on high dose of CDKI at day 14. We examined the HCV viral titers, CDK mRNA levels, and CDK protein levels in the liver tissues of three experimental groups (Supplementary Fig. 4). These results indicate that HCV titers in the liver of chimeric mice are clearly correlated with those in the serum at day 14, and that the mRNAs and proteins of CDK2, CDK4, and CDK6 are expressed in the end-point liver. These findings demonstrate that CDK inhibition suppresses HCV replication *in vivo*, suggesting that compounds of this class are candidate therapies for the treatment of HCV-infected individuals.

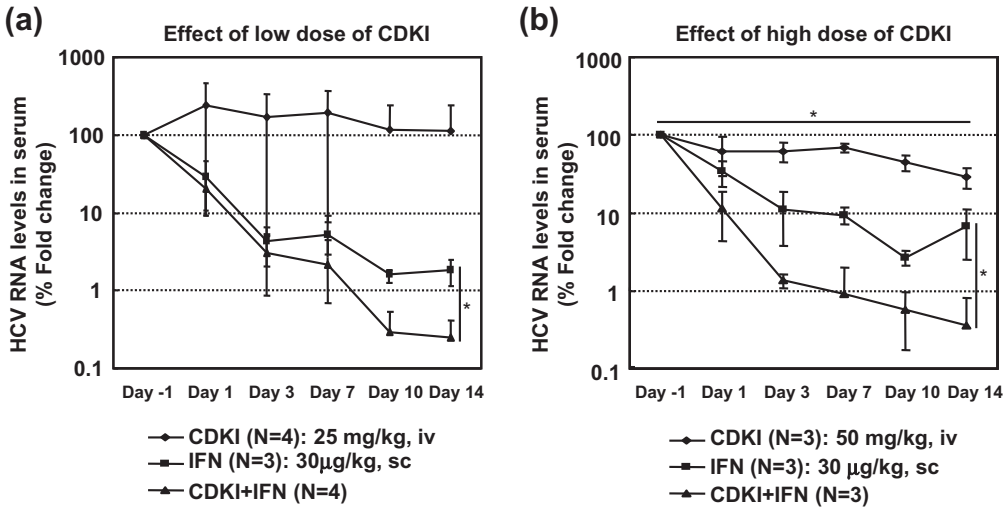

Fig. 6. *In vivo* anti-HCV activity of a CDK inhibitor. CDK inhibitor A (CDKI) and PEG-interferon α (IFN) were administered as single and combined therapies to HCV-infected chimeric mice with humanized livers. The results for low doses of CDKI are shown in (a), and those for high doses of CDKI are shown in (b). At the indicated time points, blood was sampled from each mouse. RNAs were prepared from the resulting sera and HCV RNA titers were measured by real-time RT-PCR. Each data point represents the mean ± SD from at least three mice. $^*P < 0.05$.

4. Discussion

4.1. Molecular mechanisms for suppression of HCV replication by CDK inhibitors

CDKs play important roles in the regulation of the cell cycle, a process that is perturbed in many tumor cells. Inhibition of CDK activity has therefore been proposed as a strategy for the control of cancer development and the curing of cells with abnormal growth properties (Krystof and Uldrijan, 2010). In this paper, we report that small-molecule CDK inhibitors suppress HCV replication *in vitro* and *in vivo*. We also demonstrate that interphase CDKs, including CDK4, are required for the efficient replication of HCV. Previous reports have shown that HCV genome replication is strongly dependent on cellular proliferation, and that the activity of the HCV internal ribosomal entry site (IRES) is greatest in actively growing cells (Honda et al., 2000; Scholle et al., 2004). Thus, it is possible that regulation of cell cycle in HCV-infected cells by CDK inhibitors is responsible for the suppression of HCV replication. Alternatively, CDKs may regulate HCV replication via the Rb protein. Rb, which is a direct target of CDKs, is known to bind to and inhibit HCV NS5B polymerase (Munakata et al., 2005); CDK inhibitors might activate Rb by enhancing and maintaining the phosphorylated state of Rb. Indeed, hyper-phosphorylation of Rb is reported to inhibit the LxCxE-binding activity of Rb (Knudsen and Wang, 1996), which also mediates the interaction between Rb and NS5B.

4.2. Roles of CDK4 in HCV replication

Among the CDK inhibitors tested in cell culture, a CDK4 inhibitor was the most effective in suppressing HCV replication, and RNAi analysis clearly showed that knockdown of CDK4 inhibited HCV replication. These data suggest that CDK4 is a novel host factor that regulates the HCV life cycle. CDK4 was originally identified as a catalytic partner of D-type cyclins (Matsushime et al., 1992). However, in contrast to our results for CDK4, we found that changes in cyclin D1 levels did not alter HCV replication. Knockdown and over-production of cyclin D1 appeared not to affect the regulation of HCV replication. We hypothesize that any cyclin D-specific regulation of HCV depends on some cyclin D activity distinct from protein level, or that the CDK4 dependence of HCV is mediated through a distinct binding partner in HCV-infected hepatocytes.

Knockout analysis in mice revealed that CDK4 is not essential for G1-phase progression, although CDK4-deficient mice exhibit a decreased growth rate and infertility (Rane et al., 1999). CDK4 is also suggested to play important roles in thymocyte maturation and pancreatic β-cell development (Chow et al., 2010). Thus, the CDK4-dependent aspects of HCV replication may reflect the functions of CDK4 other than cell-cycle regulation (Fig. 3b), consistent with the redundancy of CDK4 and CDK6 as interphase CDKs (Malumbres and Barbacid, 2009). At the same time, our data indicate that CDK2, CDK4, and CDK6 are each required for HCV replication in cultured cells, thus suggesting the significance of complete interphase progression during HCV replication. Normal hepatocytes are non-growing quiescent G0 cells, and enter the cell cycle after viral infection or during liver regeneration. The detailed molecular mechanisms underlying regulation of the interphase following HCV infection clearly deserves further investigation.

Interestingly, CDK4 has been demonstrated as a host factor required for HCV entry, using functional siRNA kinase screening (Lupberger et al., 2011). As our results clearly indicate that CDK inhibitors suppress HCV replication in the subgenomic replicon systems, the role of CDKs in our hypothesis is independent of HCV entry. Therefore, CDKs must have at least two distinct functions for supporting HCV replication and infection.

4.3. Relationship between endogenous CDK inhibitors and HCV replication

The presence of endogenous CDK inhibitors, such as p16, p21 and p27, helps normal hepatocytes to regulate the cell cycle (Kato et al., 1994; Toyoshima and Hunter, 1994). Production of these endogenous inhibitors is tightly regulated at every step from transcription to protein stability. Typically, p21 and p27 form a complex with CDK2 or CDK4 in quiescent hepatocytes, resulting in inhibition of kinase activity (Kwon et al., 2002). In place of cellular inhibitors, we used synthetic small-molecule inhibitors of CDKs to impair CDK function and suppress HCV replication. Given that CDK4 is required for HCV replication, up-regulation of p21 and/or p27 may be able to substitute the addition of exogenous CDK inhibitors. Indeed, HCV core is reported to stabilize p27 to arrest cell-cycle progression, and enhanced production of p21 is observed in hepatocytes of mice transgenic for HCV core (Chang et al., 2009; Yao et al., 2003). The potential role of p21 and p27 in HCV replication will require further analysis.

4.4. Conclusion

In conclusion, a novel host factor, CDK4, was identified as a potential target of HCV therapy. Small-molecule inhibitors of CDK, currently under development as anti-cancer therapies, may also find application in the treatment of individuals infected with HCV, particularly for viral genotypes 1 and 2. The combined administration of IFN and a CDK inhibitor was more effective than either alone, suggesting a possible future regimen for HCV treatment.

Acknowledgments

We are grateful to the members of our laboratories for helpful discussion and critical reading of the manuscript. This work was supported by a grant from the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan (to TM and AN), and by a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (to TM).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.05.011.

References

Barnard, R.J., McHale, C.M., Newhard, W., Cheney, C.A., Graham, D.J., Himmelberger, A.L., Strizki, J., Hwang, P.M., Rivera, A.A., Reeves, J.D., Nickle, D., Dinubile, M.J., Hazuda, D.J., Mobashery, N., 2013. Emergence of resistance-associated variants after failed triple therapy with vaniprevir in treatment-experienced non-cirrhotic patients with hepatitis C-genotype 1 infection: a population and clonal analysis. Virology 443, 278–284.

Buchkovich, K., Duffy, L.A., Harlow, E., 1989. The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58, 1097–1105.

Chang, M.L., Chen, T.H., Chang, M.Y., Yeh, C.T., 2009. Cell cycle perturbation in the hepatocytes of HCV core transgenic mice following common bile duct ligation is associated with enhanced p21 expression. J. Med. Virol. 81, 467–472.

Chellappan, S.P., Hiebert, S., Mudryj, M., Horowitz, J.M., Nevins, J.R., 1991. The E2F transcription factor is a cellular target for the RB protein. Cell 65, 1053.

Chen, H.Z., Tsai, S.Y., Leone, G., 2009. Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat. Rev. Cancer 9, 785–797.

Chow, Y.H., Zhu, X.D., Liu, L., Schwartz, B.R., Huang, X.Z., Harlan, J.M., Schnapp, L.M., 2010. Role of Cdk4 in lymphocyte function and allergen response. Cell Cycle 9, 4922–4930.

De Francesco, R., Migliaccio, G., 2005. Challenges and successes in developing new therapies for hepatitis C. Nature 436, 953–960.
Elowe, S., 2011. Bub1 and BubR1: at the interface between chromosome attachment and the spindle checkpoint. Mol. Cell. Biol. 31, 3085–3093.
Feld, J.J., Hoofnagle, J.H., 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 436, 967–972.
Honda, M., Kaneko, S., Matsushita, E., Kobayashi, K., Abell, G.A., Lemon, S.M., 2000. Cell cycle regulation of hepatitis C virus internal ribosomal entry site-directed translation. Gastroenterology 118, 152–162.
Ikeda, M., Yi, M., Li, K., Lemon, S.M., 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of the HCV-N strain of hepatitis C virus replicate efficiently in cultured Huh7 cells. J. Virol. 76, 2997–3006.
Kato, J.-y., Matsuoka, M., Polyak, K., Massague, J., Sherr, C.J., 1994. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. Cell 79, 487.
Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125, 1808–1817.
Keeffe, E.B., 2007. Future treatment of chronic hepatitis C. Antivir. Ther. 12, 1015–1025.
Khidr, L., Chen, P.L., 2006. RB, the conductor that orchestrates life, death and differentiation. Oncogene 25, 5210–5219.
Knudsen, E.S., Wang, J.Y., 1996. Differential regulation of retinoblastoma protein function by specific Cdk phosphorylation sites. J. Biol. Chem. 271, 8313–8320.
Knudson Jr., A.G., 1971. Mutation and cancer: statistical study of retinoblastoma. Proc. Natl. Acad. Sci. U.S.A. 68, 820–823.
Krystof, V., Uldrijan, S., 2010. Cyclin-dependent kinase inhibitors as anticancer drugs. Curr. Drug Targets 11, 291–302.
Kwon, Y.H., Jovanovic, A., Serfas, M.S., Kiyokawa, H., Tyner, A.L., 2002. P21 functions to maintain quiescence of p27-deficient hepatocytes. J. Biol. Chem. 277, 41417–41422.
Lamarre, D., Anderson, P.C., Bailey, M., Beaulieu, P., Bolger, G., Bonneau, P., Bos, M., Cameron, D.R., Cartier, M., Cordingley, M.G., Faucher, A.M., Goudreau, N., Kawai, S.H., Kukolj, G., Lagace, L., LaPlante, S.R., Narjes, H., Poupart, M.A., Rancourt, J., Sentjens, R.E., St George, R., Simoneau, B., Steinmann, G., Thibeault, D., Tsantrizos, Y.S., Weldon, S.M., Yong, C.L., Llinas-Brunet, M., 2003. An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature 426, 186–189.
Lin, C., Gates, C.A., Rao, B.G., Brennan, D.L., Fulghum, J.R., Luong, Y.P., Frantz, J.D., Lin, K., Ma, S., Wei, Y.Y., Perni, R.B., Kwong, A.D., 2005. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J. Biol. Chem. 280, 36784–36791.
Lupberger, J., Zeisel, M.B., Xiao, F., Thumann, C., Fofana, I., Zona, L., Davis, C., Mee, C.J., Turek, M., Gorke, S., Royer, C., Fischer, B., Zahid, M.N., Lavillette, D., Fresquet, J., Cosset, F.L., Rothenberg, S.M., Pietschmann, T., Patel, A.H., Pessaux, P., Doffoel, M., Raffelsberger, W., Poch, O., McKeating, J.A., Brino, L., Baumert, T.F., 2011. EGFR and EphA2 are host factors for hepatitis C virus entry and possible targets for antiviral therapy. Nat. Med. 17, 589–595.
Macartney, M.J., Irish, D., Bridge, S.H., Garcia-Diaz, A., Booth, C.L., McCormick, A.L., Labbett, W., Smith, C., Velazquez, C., Tanwar, S., Trembling, P., Jacobs, M., Dusheiko, G., Rosenberg, W., Haque, T., 2014. Telaprevir or boceprevir based therapy for chronic hepatitis C infection: development of resistance-associated variants in treatment failure. Antiviral Res. 105c, 112–117.
Malumbres, M., Barbacid, M., 2009. Cell cycle, CDKs and cancer: a changing paradigm. Nat. Rev. Cancer 9, 153–166.
Manns, M.P., Gane, E., Rodriguez-Torres, M., Stoehr, A., Yeh, C.T., Marcellin, P., Wiedmann, R.T., Hwang, P.M., Caro, L., Barnard, R.J., Lee, A.W., 2012. Vaniprevir with pegylated interferon alpha-2a and ribavirin in treatment-naive patients with chronic hepatitis C: a randomized phase II study. Hepatology 56, 884–893.
Matsushime, H., Ewen, M.E., Strom, D.K., Kato, J.Y., Hanks, S.K., Roussel, M.F., Sherr, C.J., 1992. Identification and properties of an atypical catalytic subunit (p34PSK-J3/cdk4) for mammalian D type G1 cyclins. Cell 71, 323–334.
Mayhew, C.N., Carter, S.L., Fox, S.R., Sexton, C.R., Reed, C.A., Srinivasan, S.V., Liu, X., Wikenheiser-Brokamp, K., Boivin, G.P., Lee, J.S., Aronow, B.J., Thorgeirsson, S.S., Knudsen, E.S., 2007. RB loss abrogates cell cycle control and genome integrity to promote liver tumorigenesis. Gastroenterology 133, 976–984.
McClue, S.J., Blake, D., Clarke, R., Cowan, A., Cummings, L., Fischer, P.M., MacKenzie, M., Melville, J., Stewart, K., Wang, S., Zhelev, N., Zheleva, D., Lane, D.P., 2002. In vitro and in vivo antitumor properties of the cyclin dependent kinase inhibitor CYC202 (R-roscovitine). Int. J. Cancer 102, 463–468.
McClue, S.J., Stuart, I., 2008. Metabolism of the trisubstituted purine cyclin-dependent kinase inhibitor seliciclib (R-roscovitine) in vitro and in vivo. Drug Metab. Dispos. 36, 561–570.
Meijer, L., Borgne, A., Mulner, O., Chong, J.P., Blow, J.J., Inagaki, N., Inagaki, M., Delcros, J.G., Moulinoux, J.P., 1997. Biochemical and cellular effects of roscovitine, a potent and selective inhibitor of the cyclin-dependent kinases cdc2, cdk2 and cdk5. Eur. J. Biochem. 243, 527–536.
Munakata, T., Liang, Y., Kim, S., McGivern, D.R., Huibregtse, J., Nomoto, A., Lemon, S.M., 2007. Hepatitis C virus induces E6AP-dependent degradation of the retinoblastoma protein. PLoS Pathog. 3, 1335–1347.
Munakata, T., Nakamura, M., Liang, Y., Li, K., Lemon, S.M., 2005. Down-regulation of the retinoblastoma tumor suppressor by the hepatitis C virus NS5B RNA-dependent RNA polymerase. Proc. Natl. Acad. Sci. U.S.A. 102, 18159–18164.
Musgrove, E.A., Caldon, C.E., Barraclough, J., Stone, A., Sutherland, R.L., 2011. Cyclin D as a therapeutic target in cancer. Nat. Rev. Cancer 11, 558–572.
Nakagawa, S., Umehara, T., Matsuda, C., Kuge, S., Sudoh, M., Kohara, M., 2007. Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice. Biochem. Biophys. Res. Commun. 353, 882–888.
Nutley, B.P., Raynaud, F.I., Wilson, S.C., Fischer, P.M., Hayes, A., Goddard, P.M., McClue, S.J., Jarman, M., Lane, D.P., Workman, P., 2005. Metabolism and pharmacokinetics of the cyclin-dependent kinase inhibitor R-roscovitine in the mouse. Mol. Cancer Ther. 4, 125–139.
Pawlotsky, J.-M., 2009. Hepatitis: HCV variability, the immune system and resistance to antiviral drugs. Nat. Rev. Gastroenterol. Hepatol. 6, 383.
Poordad, F., Lawitz, E., Reddy, K.R., Afdhal, N.H., Hezode, C., Zeuzem, S., Lee, S.S., Calleja, J.L., Brown Jr., R.S., Craxi, A., Wedemeyer, H., Nyberg, L., Nelson, D.R., Rossaro, L., Balart, L., Morgan, T.R., Bacon, B.R., Flamm, S.L., Kowdley, K.V., Deng, W., Koury, K.J., Pedicone, L.D., Dutko, F.J., Burroughs, M.H., Alves, K., Wahl, J., Brass, C.A., Albrecht, J.K., Sulkowski, M.S., 2013. Effects of ribavirin dose reduction vs erythropoietin for boceprevir-related anemia in patients with chronic hepatitis C virus genotype 1 infection – a randomized trial. Gastroenterology 145, 1035–1044.e1035.
Rane, S.G., Dubus, P., Mettus, R.V., Galbreath, E.J., Boden, G., Reddy, E.P., Barbacid, M., 1999. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. Nat. Genet. 22, 44–52.
Reed, C.A., Mayhew, C.N., McClendon, A.K., Knudsen, E.S., 2010. Unique impact of RB loss on hepatic proliferation: tumorigenic stresses uncover distinct pathways of cell cycle control. J. Biol. Chem. 285, 1089–1096.
Sarrazin, C., Kieffer, T.L., Bartels, D., Hanzelka, B., Muh, U., Welker, M., Wincheringer, D., Zhou, Y., Chu, H.M., Lin, C., Weegink, C., Reesink, H., Zeuzem, S., Kwong, A.D., 2007. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 132, 1767–1777.
Scholle, F., Li, K., Bodola, F., Ikeda, M., Luxon, B.A., Lemon, S.M., 2004. Virus-host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis. J. Virol. 78, 1513–1524.
Simanis, V., Nurse, P., 1986. The cell cycle control gene cdc2+ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. Cell 45, 261–268.
Simmonds, P., Bukh, J., Combet, C., Deleage, G., Enomoto, N., Feinstone, S., Halfon, P., Inchauspe, G., Kuiken, C., Maertens, G., Mizokami, M., Murphy, D.G., Okamoto, H., Pawlotsky, J.M., Penin, F., Sablon, E., Shin, I.T., Stuyver, L.J., Thiel, H.J., Viazov, S., Weiner, A.J., Widell, A., 2005. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology 42, 962–973.
Takeuchi, T., Katsume, A., Tanaka, T., Abe, A., Inoue, K., Tsukiyama-Kohara, K., Kawaguchi, R., Tanaka, S., Kohara, M., 1999. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 116, 636–642.
Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T., Yoshizato, K., 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am. J. Pathol. 165, 901–912.
Teramoto, N., Pokrovskaja, K., Szekely, L., Polack, A., Yoshino, T., Akagi, T., Klein, G., 1999. Expression of cyclin D2 and D3 in lymphoid lesions. Int. J. Cancer 81, 543–550.
Toyoshima, H., Hunter, T., 1994. P27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. Cell 78, 67–74.
Uhlmann, F., Bouchoux, C., Lopez-Aviles, S., 2011. A quantitative model for cyclin-dependent kinase control of the cell cycle: revisited. Philos. Trans. R. Soc. Lond. B Biol. Sci. 366, 3572–3583.
Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11, 791–796.
Waters, L., Nelson, M., 2006. New therapeutic options for hepatitis C. Curr. Opin. Infect. Dis. 19, 615–622.
Whittaker, S.R., Walton, M.I., Garrett, M.D., Workman, P., 2004. The cyclin-dependent kinase inhibitor CYC202 (R-roscovitine) inhibits retinoblastoma protein phosphorylation, causes loss of cyclin D1, and activates the mitogen-activated protein kinase pathway. Cancer Res. 64, 262–272.
Yao, Z.Q., Eisen-Vandervelde, A., Ray, S., Hahn, Y.S., 2003. HCV core/gC1qR interaction arrests T cell cycle progression through stabilization of the cell cycle inhibitor p27Kip1. Virology 314, 271–282.