Biochemical and Biophysical Research Communications 421 (2012) 112–118

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Destabilization of PDK1 by Hsp90 inactivation suppresses hepatitis C virus replication through inhibition of PRK2-mediated viral RNA polymerase phosphorylation

Mi-Gyeong Kim, Jae-Su Moon, Eun-Jung Kim, Seung-Hoon Lee, Jong-Won Oh *

Department of Biotechnology and Translational Research Center for Protein Function Control, Yonsei University, Seoul 120-749, Republic of Korea

ARTICLE INFO

Article history:
Received 20 March 2012
Available online 3 April 2012

Keywords:
Hsp90 inhibitor
17-DMAG
HCV
PDK1
RNA-dependent RNA polymerase

ABSTRACT

Heat shock protein 90 (Hsp90), which chaperones multiple client proteins, has been shown to be implicated in HCV replication. Pharmacological inhibitors of Hsp90 display an anti-HCV activity. However, little is known about the mechanisms of regulation of HCV replication by Hsp90. Here, we show that Hsp90 inhibition by 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG) destabilizes phosphoinositide-dependent kinase-1 (PDK1), an upstream kinase of the protein kinase C-related kinase 2 (PRK2) responsible for phosphorylation of HCV RNA polymerase, through the proteosome pathway. Destabilization of PDK1 led to inhibition of phosphorylation of the viral RNA polymerase through a decrease in the abundance of active form PRK2 level. Consequently, Hsp90 inhibition resulted in suppression of HCV replication both in human hepatoma Huh7 cells harboring an HCV subgenomic replicon and in HCV-infected cells. 17-DMAG treatment did not interfere with HCV internal ribosome entry site-mediated translation and the cell cycle in Huh7 cells. Co-treatment of 17-DMAG with interferon-α or HA1077, an inhibitor of PRK2, enhanced the anti-HCV activity of 17-DMAG. Taken together, these findings suggest that Hsp90 plays a critical role in the regulation of HCV RNA polymerase phosphorylation via the PDK1–PRK2 signaling pathway.

1. Introduction

Hepatitis C virus (HCV) is the major etiologic agent of non-A and non-B hepatitis. More than 170 million people worldwide are chronically infected with HCV. Persistent HCV infection establishes a chronic hepatitis often leading to liver cirrhosis and hepatocellular carcinoma (HCC) [1]. Currently, effective, HCV-specific therapeutic agents are rather limited. The standard HCV therapy, a combination of pegylated interferon (IFN)-α and ribavirin, is associated with various adverse effects. Furthermore, IFN-α is effective in less than 50% of genotype 1 HCV-infected patients [2]. Therefore, various therapeutic options have been under investigation in order to develop effective and specific therapies with the least toxicity.

HCV has a positive sense single-strand RNA genome of approximately 9.6 kb, which consists of one long open reading frame (ORF) flanked by untranslated regions (UTRs) at both the 5′ and 3′ ends of the genome. The ORF encodes a single polyprotein that is proteolytically processed by cellular and viral proteases into at least 10 functional viral proteins, including both structural and nonstructural (NS) proteins [3]. The 65-kDa HCV NS5B protein, responsible for RNA-dependent RNA polymerase (RdRp) activity, is the key enzyme essential for HCV RNA replication. In our previous studies, we showed that this viral RNA polymerase is phosphorylated by protein kinase C-related kinase 2 (PRK2) [4] and that inhibition of PRK2 activity by PRK2 inhibitors effectively suppresses HCV replication [5].

Heat shock protein 90 (Hsp90), which helps folding of multiple client proteins, displays pleiotropic functions through its interaction with various cellular client proteins, protecting them from proteosomal degradation [6]. Hsp90 has also been identified as an essential host-factor in the maturation of viral proteins [7,8]. Recently, it has been demonstrated that Hsp90 forms a trimeric complex with HCV NS5A and FKBP8, a cyclophilin family immunophilin, to regulate HCV RNA replication [9]. Furthermore, the Hsp90 inhibitors 17-allyaminogeldanamycin (17-AAG) and 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), analogs of the benzoquinone ansamycin antibiotic geldanamycin, were shown to inhibit HCV replication in cell-based assays using HCV replicons and in HCV-infected chimeric mice with *trans*-humanized liver

Abbreviations: Hsp90, heat shock protein 90; HCV, hepatitis C virus; RdRp, RNA-dependent RNA polymerase; 17-DMAG, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin; PDK1, phosphoinositide-dependent kinase-1; PRK2, protein kinase C-related kinase 2.

* Corresponding author. Fax: +82 2 362 7265.
E-mail address: jwoh@yonsei.ac.kr (J.-W. Oh).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.03.126

[9,10]. To date, however, the precise role of Hsp90 in HCV replication remains unknown. One of potential Hsp90 clients is the 3-phosphoinositide-dependent kinase-1 (PDK1) responsible for PRK2 activation [4], raising the question of whether Hsp90 inhibition leads to an inhibitory effect on HCV replication by affecting the phosphorylation of HCV NS5B RdRp.

In the present study, we show that inactivation of Hsp90 interferes with the PDK1–PRK2 signaling pathway by promoting the degradation of PDK1. We also demonstrate that reduction in the active form PRK2 level through PDK1 destabilization results in abrogation of HCV NS5B phosphorylation, leading to suppression of HCV replication.

2. Material and methods

2.1. Cell culture and reagents

The human hepatoma cell line Huh7 was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin, and 1% nonessential amino acids under standard culture conditions (5% CO_2, 37 °C). The human hepatoma stable cell lines Huh7TR-4 and Huh7TR-NS [4], which express the tetracycline repressor and the HCV NS proteins (NS3–NS5) in a tetracycline-inducible manner, respectively, were maintained in the presence of blasticidin S (10 μg/ml) and Zeocine (100 μg/ml). The Huh7 cell-derived cell line R-1, supporting stable, autonomous replication of genotype 1b HCV subgenomic replicon RNA, was described previously [4]. 17-DMAG, interferon (IFN)-αA (I-4276), and the proteosome inhibitor benzyloxycarbonyl-Leu-Leu-Leu aldehyde (MG132) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The PRK2 inhibitor HA1077 was from Calbiochem Inc. (La Jolla, CA, USA) and the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002 from Biomol (Plymouth Meeting, PA, USA).

2.2. HCV infection and drug treatment

Full-length, infectious HCV RNA of the genotype 2a HCV clone JFH1 was prepared by in vitro transcription using the MEGAscript T7 kit (Ambion, Austin, TX, USA) and electroporated into Huh7 cells to obtain cell culture-derived HCV (HCVcc) as described previously [11]. Huh7 cells were infected with the HCVcc at a multiplicity of infection (MOI) of 0.3 by adsorption for 12 h with periodic rocking, then washed three times with phosphate-buffered saline (PBS), and maintained in complete DMEM as described previously [12]. R-1 cells or Huh7 cells infected with HCVcc were treated with 17-DMAG or PRK2 inhibitor alone or in combination with IFN-α (100 IU/ml) for 72 h. The half maximal inhibitory concentration (IC_{50}) value for 17-DMAG to inhibit HCV replication in Huh7 cells was determined by fitting data to a four-parametric sigmoidal function using SigmaPlot (Version 10.0; Systat Software, Richmond, CA, USA).

2.3. Cytotoxicity assay

The cytotoxicity of 17-DMAG was measured using MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxylmethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] reagent (Promega, Madison, WI, USA) according to the manufacturer's protocol as described previously [13].

2.4. Western blot analysis and immunoprecipitation

Total cell lysates from cells treated with inhibitors or IFN-α were used for Western blot analysis, as described previously [4], using antibodies against PDK1, p-PDK1(S241), PRK2, and p-PRK2(T816) from Cell Signaling Technology (Beverly, MA, USA). A polyclonal antibody against a consensus sequence present in the three protein kinase C (PKC) isoforms (α, β, γ) (anti-pan-PKC; Zymed Laboratories, San Francisco, CA, USA) was used to detect PKC isoforms (α, β, γ). Mouse monoclonal anti-Hsp90 antibody AC88 was from StressGen (Victoria, BC, Canada). An anti-α-tubulin antibody (Oncogene Research Products, Cambridge, MA, USA) was used to verify equal protein loading. Immunoprecipitation experiments for detection of phosphorylated form NS5B was performed using an anti-p-Ser antibody (clone PSR-45; Sigma–Aldrich) as described previously [4]. Immunoprecipitates, resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), were transferred to nitrocellulose membranes (Hybond-ECL; Amersham Biosciences, Piscataway, NJ, USA) and immunoblotted with affinity-purified anti-NS5B polyclonal antibodies [5].

2.5. Real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was extracted from R-1 or HCV (JFH1)-infected cells using TRIzol LS reagent (Invitrogen, Carlsbad, CA, USA) and purified according to the manufacturer's instructions. HCV RNA levels were quantified by RT-PCR using a primer pair targeting a region within the HCV 5′-UTR and TaqMan probe as described previously [4]. qRT-PCR analysis of PDK1 mRNA level was performed using SYBR Premix ExTaq (Takara, Japan) and specific oligonucleotide primers for target sequences, as well as the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. The following primers were used: PDK1: sense 5′-GAACGAGCCTACGTGTGTACCTGA-3′, antisense 5′-ATTCTGCCAGGTGGTTAGGCTATG-3′; GAPDH: sense 5′-GAAGGTGAAGGTCGGAGTC-3′, antisense 5′-GAAGATGGTGATGGGATTTC-3′. Target gene levels normalized to GAPDH were determined using the ΔΔCt method, as described previously [14].

2.6. Dual luciferase reporter assay

The dual luciferase expression vector allowing cap-dependent translation of a *Renilla* luciferase (RLuc) reporter and HCV internal ribosome entry site (IRES)-mediated translation of a firefly luciferase (FLuc) reporter was constructed by inserting the HCV IRES and partial core protein-coding gene fused to the cDNA for FLuc downstream of the RLuc reporter gene as described previously [15]. Huh7 cells were transiently transfected with the bicistronic dual luciferase vector using Fugene HD (Roche Applied Science, Indianapolis, IN, USA). RLuc and FLuc activities in cell lysates were quantified using the Dual-Glo luciferase assay system and a GloMax-Multi detection system (Promega).

2.7. Cell cycle analysis

After exposure of Huh7 cells to drugs, cells were washed with PBS and fixed with chilled ethanol for 30 min. Cells were then treated with RNase A (100 μg/ml) for 5 min at room temperature, stained with 50 μg/ml propidium iodide (Sigma–Aldrich) for 30 min at 37 °C, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

3. Results

3.1. Destabilization of PDK1 by 17-DMAG in Huh7cells

Previously, Hsp90 inhibition using geldanamycin was shown to destabilize PDK1 in HEK293 cells [16]. This destabilization effect, however, was not observed in breast cancer cell lines [17]. Because

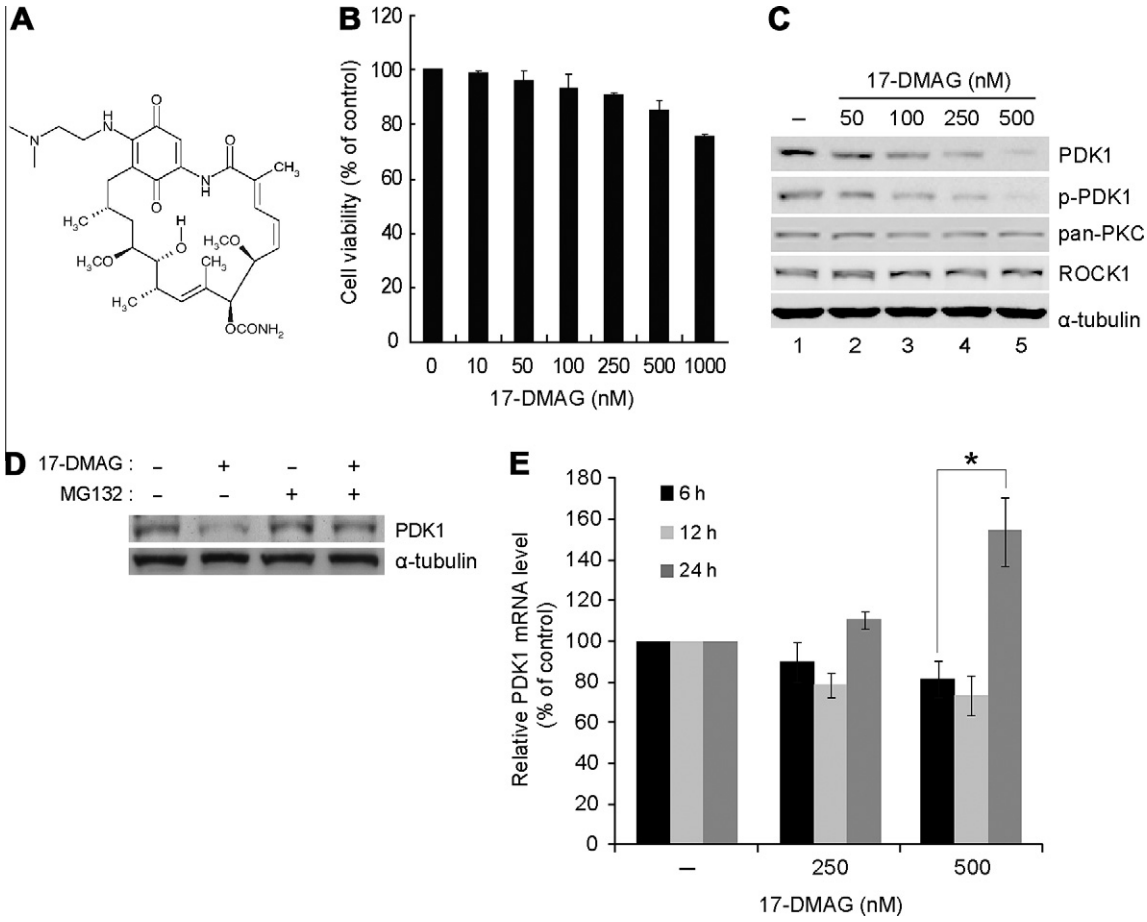

Fig. 1. Destabilization of PDK1 via the proteosome-dependent degradation pathway. (A) Chemical structure of 17-DMAG. (B) Huh7 cells were treated with the indicated concentrations of 17-DMAG for 72 h before assessing cell viability by the MTS assay. (C) Huh7 cells treated with 17-DMAG for 48 h were analyzed by Western blot analyses for the indicated proteins. (D) Huh7 cells treated with 250 nM 17-DMAG with and without 10 mM MG132 for 18 h were analyzed for the indicated proteins by Western blot analyses. (E) Huh7 cells treated with indicated concentrations of 17-DMAG were analyzed for PDK1 mRNA abundance by real-time qRT-PCR. Relative mRNA abundance is shown. Data represent mean ± standard deviation (SD) of triplicate measurements from three independent experiments. $^{*}P < 0.05$ (Student's *t*-test).

PDK1 is the upstream kinase of the PRK2 phosphorylating the HCV NS5B RdRp [4], we were interested in investigating whether PDK1 is destabilized by 17-DMAG in the human hepatoma cell line Huh7 supporting HCV replication. The Hsp90 inhibitor 17-DMAG (Fig. 1A) used in this work is a second generation geldanamycin derivative known to be effective in oral delivery and safer than geldanamycin or its analog 17-AAG [6,18]. We first examined the cell viability of Huh7 cells after treatment with 17-DMAG (0–1 mM), using the MTS assay. In this cell line, 17-DMAG did not significantly affect cell viability at concentrations below 500 nM (Fig. 1B). Thus, this dose was selected as the maximal concentration for the subsequent studies. Western blot analysis revealed that 17-DMAG decreases the abundance of PDK1 gradually and significantly in Huh7 cells treated with increasing concentrations of 17-DMAG (upto 500 nM 17-DMAG) for 48 h, whereas the levels of other kinases, such as pan-PKC (α, β, γ) and Rho-kinase I (ROCKI), remained unchanged (Fig. 1C). The decrease of PDK1 abundance by Hsp90 inhibition with 17-DMAG could be rescued by co-treatment with the proteosome inhibitor MG132 (Fig. 1D). Of note, there was no significant decrease in the PDK1 mRNA level by Hsp90 inhibition (Fig. 1E). Instead, we reproducibly observed a slight increase of PDK1 mRNA level particularly in cells treated with 500 nM 17 DMAG for 24 h, which did not, however, influence the PDK1 destabilization effect of 17-DMAG even at 48 h post-treatment (Fig. 1C, lane 5). Thus, these results altogether suggest that the reduction in PDK1 abundance by 17-DMAG treatment is mediated by destabilization of PDK1 via the proteosome-dependent pathway.

3.2. Interference of PDK1–PRK2 signaling pathway by 17-DMAG inhibits HCV NS5B phosphorylation

PRK2 was previously shown to phosphorylate HCV NS5B to enhance HCV replication [4,5]. Since the activation of PRK2 is mediated by PDK1, we sought to test whether 17-DMAG affects the NS5B phosphorylation. To this end, we first evaluated the cellular toxicity of 17-DMAG in the R-1 cells harboring a selectable HCV subgenomic replicon. In a dramatic contrast to the results observed with the parental cell line of R-1, Huh7, 17-DMAG significantly reduced cell viability at the concentrations over 50 nM (Fig. 2A). Thus, we treated the R-1 cells with less than 50 nM of 17-DMAG to measure its ability to destabilize the PDK1. As demonstrated by Western blot analysis in the results shown in Fig. 2B, there was approximately 40% reduction in the abundance of PDK1 after treatment with 50 nM 17-DMAG (Fig. 2B). The decrease of PDK1 level was accompanied by a concomitant decrease of its active phosphorylated form; S241 phosphorylation known to be crucial for PDK1 activity [19] decreased by 57%. Abundance of PRK2 active form p-PRK2(T816) was also decreased while PRK2 level remained unchanged. As shown in Fig. 2C, ~50% reduction of the p-PRK2(T816) level was associated with inhibition of HCV replication by 48% as assessed by real-time qRT-PCR analysis for HCV subgenomic RNA level

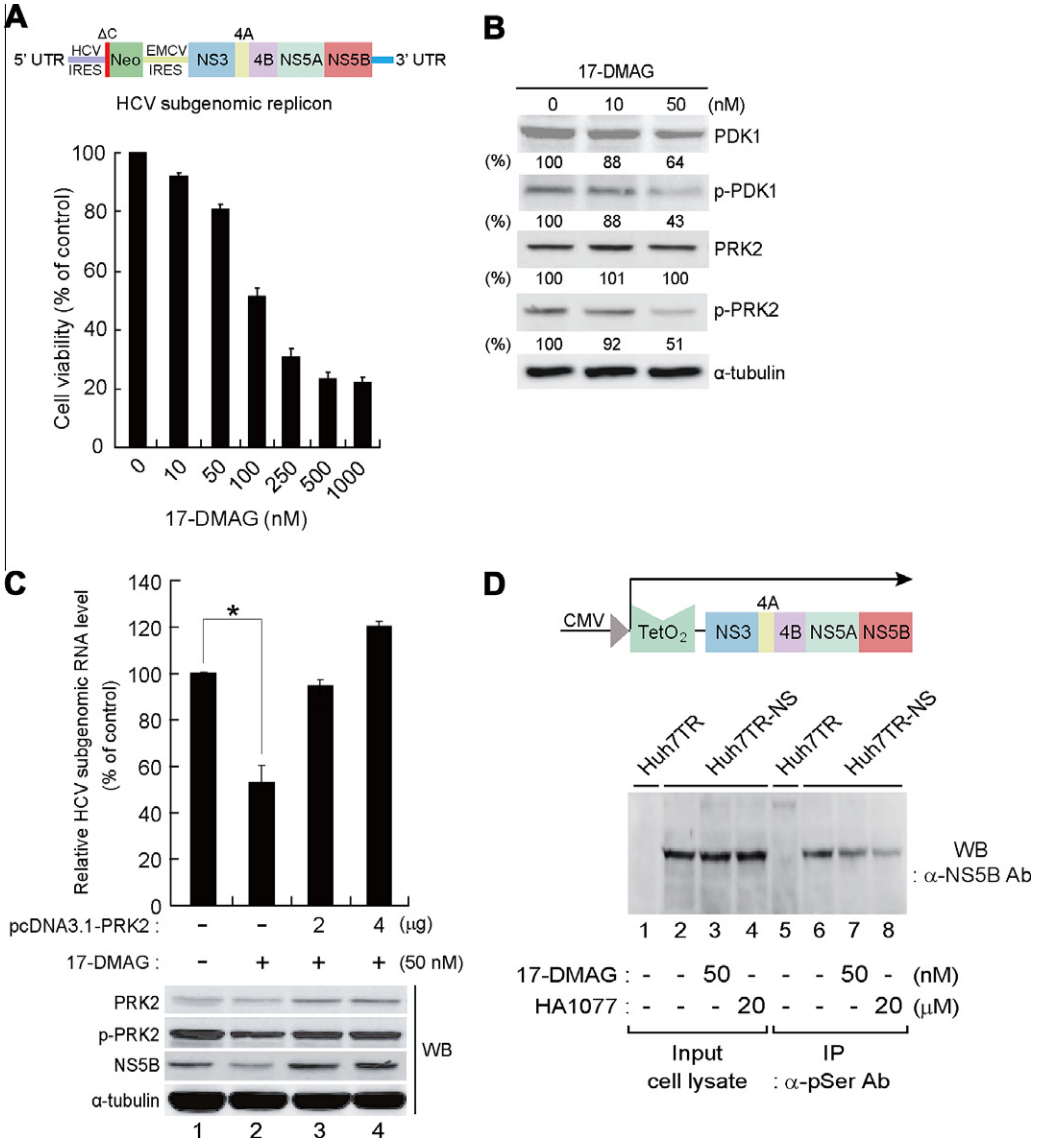

Fig. 2. Hsp90 inhibition modulates PDK1–PRK2 signaling pathway crucial for HCV NS5B phosphorylation. (A) Effect of 17-DMAG on viability of R-1 cells was assessed as in Fig. 1B. The top panel shows a schematic representation of the bicistronic selectable HCV subgenomic replicon in which ΔC and Neo represent the N-terminal part of HCV core protein and neomycin phosphotransferase, respectively. The NS proteins (NS3–NS5B), which are essential for HCV replication, are indicated in the diagram. (B) R-1 cells were treated with vehicle (0.25% DMSO) or 17-DMAG at the indicated concentrations for 72 h before immunoblotting cell lysates with antibodies specific for the indicated proteins. Amount of protein was determined by densitometric analysis of immunoblots and the relative intensities calculated for PDK1 and PRK2 as well as their active phosphorylated forms were normalized over the α-tubulin signal. A representative blot from three independent experiments showing similar results is shown. (C) R-1 cell transfected with pcDNA3.1 vector (first and second bars) or increasing amounts of pcDNA3.1-PRK2 were treated with vehicle (first bar) or 50 nM 17-DMAG. After 72 h, cells were harvested for analysis of remaining intracellular HCV RNA levels. Relative HCV genome copy numbers are expressed as percent of DMSO-treated controls. Three independent experiments were performed in triplicate. Data shown are the mean ± SD of three independent experiments. $^{*}P < 0.05$ (Student's *t*-test). Western blot (WB) analyses were performed for the indicated proteins (bottom panel). (D) Huh7TR-NS cells [schematic diagram of the Tet-inducible vector expressing HCV NS proteins (NS3–NS5B) is shown above the blot] were cultured with 1 μg/ml tetracycline for 24 h to induce expression of the NS proteins, followed by treatment with 17-DMAG (50 nM) or HA1077, a PRK2 inhibitor (20 μM) for 48 h. The Huh7TR-NS parental cell line, Huh7TR-4 not expressing the HCV NS proteins, was used as a control. Serine-phosphorylated proteins were immunoprecipitated from cell lysates using an anti-phosphoserine antibody. The immunoprecipitated proteins (lanes 5–8) and 4% of each input cell lysate (lanes 1–4) were immunoblotted with purified polyclonal anti-NS5B antibodies.

(second bar) and by Western blot analysis for NS5B level (lower panel, lane 2). Furthermore, the 17-DMAG-mediated inhibition of HCV replication could be rescued by ectopic expression of PRK2, suggesting that the replication inhibitory effect of 17-DMAG is via the PDK1–PRK2 signaling pathway.

We then analyzed by immunoprecipitation experiments the levels of phosphorylated form NS5B (p-NS5B) in the HCVTR-NS cell line. This cell line, which is defective in HCV replication but expresses HCV NS proteins (NS3–NS5B) in a tetracycline-dependent manner (Fig. 2D, upper panel), was used because 17-DMAG-mediated inhibition of HCV replication resulted in reduction of NS5B protein level in R-1 cells (Fig. 2C, bottom panel, compare lanes 1 and 2). As shown in Fig. 2D, immunoprecipitation of p-NS5B with an anti-p-Ser antibody followed by immunoblotting analysis with anti-NS5B antibody revealed that 17-DMAG treatment (50 nM) interferes with NS5B phosphorylation (compare lanes 6 and 7). Similarly, HA1077 (20 μM), a PRK2 inhibitor [4] used as a positive control, inhibited PRK2-mediated NS5B phosphorylation (lane 8). Notably, under these conditions, neither 17-DMAG or HA1077 reduced the NS5B level in the Huh7TR-NS cells (compare lane 2 with lanes 3 and 4). Taken together, the results in Fig. 2 demonstrate that PDK1 destabilization by 17-DMAG leads to inhibition of HCV

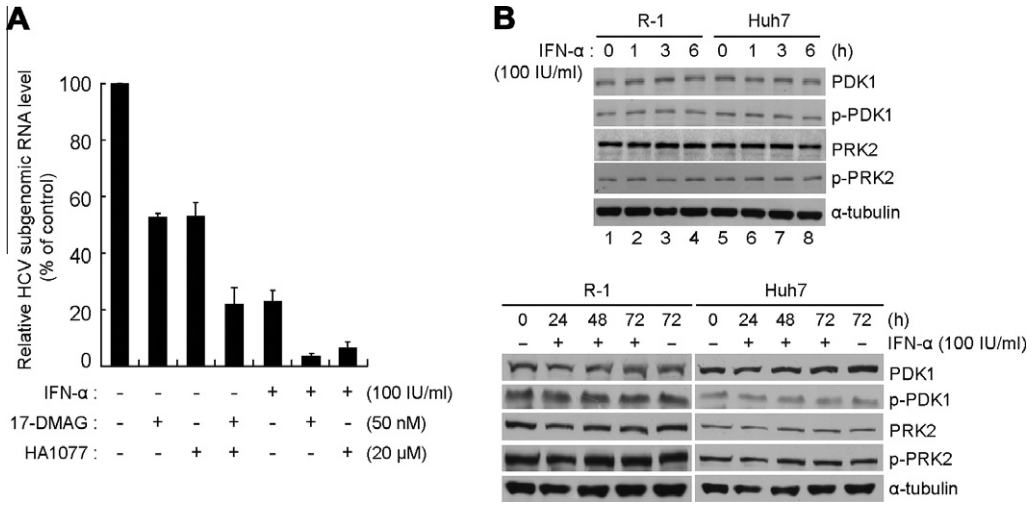

Fig. 3. Effective suppression of HCV replication by combination of 17-DMAG and IFN-α or the PRK2 inhibitor HA1077. (A) R-1 cells were treated with 50 nM 17-DMAG and/or 20 μM HA1077 alone or in combination with 100 IU/ml IFN-α for 72 h. HCV replicon RNA levels in HCV subgenomic replicon-harboring cells were determined as in Fig. 2C. (B) Effect of IFN-α on PDK1–PRK2 signaling pathway. R-1 or Huh7 cells were treated with 100 IU/ml IFN-α for the indicated times. Total cell lysates were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and immunoblotted with antibodies specific for the indicated proteins. Immunoblotting with an anti-α-tubulin antibody was used as an internal control for loading. Representative results from one of three independent experiments showing similar results are shown.

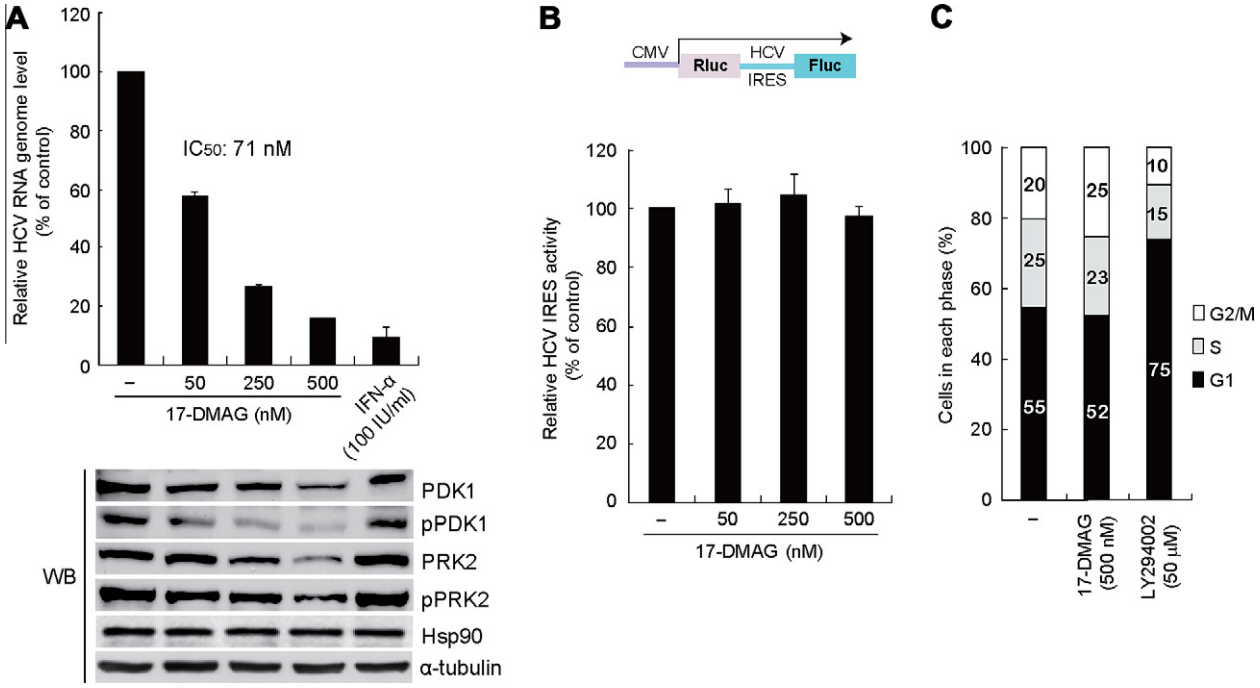

Fig. 4. Anti-HCV effect of 17-DMAG in HCV (JFH1)-infected cells. (A) Huh7 cells infected with HCV (JFH1) at an MOI of 0.3 were treated with increasing concentrations of 17-DAMG or IFN-α (100 IU/ml) for 72 h. Intracellular HCV RNA titers were quantified as in Fig. 2C. Western blot (WB) analyses were performed for the indicated proteins (bottom panel). (B) Huh7 cells transfected with the dual luciferase reporter vector (upper part) were treated with increasing concentrations of 17-DMAG for 48 h. Levels of *Renillar* luciferase (cap-dependent translation) and firefly luciferase (HCV IRES-mediated translation) activities were determined and expressed as percentage of DMSO-treated control (first bar). Three independent experiments were performed in triplicate. Data shown are the mean ± SD of three independent experiments. (C) Huh7 cells were treated with DMSO or 500 nM 17-DMAG for 48 h. Cell cycles were analyzed using a flow cytometer and percentages of G1, S, and G2/M phase cells are shown. LY294002, an inhibitor of PI3 K, was used as a positive control.

NS5B phosphorylation through the reduction of the active phosphorylated form PRK2 level.

3.3. Co-treatment with 17-DMAG and IFN-α or HA1077 effectively suppresses HCV replication

Despite various adverse effects of IFN-α, current therapy for HCV patients uses IFN-α combined with a nucleoside analog, ribavirin [2]. We evaluated the anti-HCV potency of 17-DMAG in combination with IFN-α. R-1 cells were treated with 50 nM 17-DMAG alone or in combination with IFN-α (100 IU/ml) for 72 h and the level of HCV subgenomic replicon RNA was analyzed by qRT-PCR. As shown in Fig. 3A, the combined treatment led to 96% viral RNA reduction while treatment with 100 IU/ml IFN-α alone decreased HCV RNA levels by 75%. The combination of HA1077 (20 μM) with IFN-α (100 IU/ml) also potentiated the

anti-HCV activity of IFN-α. Furthermore, 17-DMAG (50 nM) and HA1077 (20 μM) co-treatment inhibited the viral replication by 78%, the similar level of inhibition achieved with 100 IU/ml IFN-α alone, indicating that this combination is highly effective in suppressing HCV replication through blocking HCV NS5B phosphorylation.

Little is known about the role, if any, of IFN signaling pathway in regulation of PDK1 activation, expression, or stability. Thus, we next asked whether IFN-α can affect the PDK1–PRK2 signaling pathway in HCC cells. R-1 cells were treated with 100 IU/ml IFN-α for various times up to 6 h. Cell lysates were then subjected to SDS–PAGE, followed by immunoblot analysis. As shown in Fig. 3B (top panel, lanes 1–4), no significant alteration in either PDK1 or p-PDK1 levels was observed after IFN-α treatment. In addition, no change in PRK2 activation occurred in the treated cells. Similar results were also observed in Huh7 cells (lanes 5–8). Further, longer incubation with IFN-α up to 72 h did not alter the steady state levels of PDK1 and PRK2 as well as their phosphorylated forms in R-1 and Huh7 cell lines (bottom panel). These results suggest that the abundance of PDK1 and PRK2 and their activation status are not modulated by IFN-α.

3.4. Anti-HCV activity of 17-DMAG in HCV-infected cells

To evaluate the anti-HCV activity of 17-DMAG in virus-infected cells, we used an HCV infection system developed for the genotype 2a isolate JFH1, which produces infectious viruses in Huh7 [11]. Huh7 cells were infected with the cell culture-generated HCV and treated with up to 500 nM 17-DMAG. As shown in Fig. 4A, treatment with 500 nM 17-DMAG reduced the HCV RNA level by 87%, with an IC_{50} value of 71 nM. This inhibitory effect was comparable to that of IFN-α treatment (90% inhibition at 100 IU/ml). As in Huh7 cells, treatment with 17-DMAG dose-dependently decreased the levels of PDK1 and pPDK1, while Hsp90 abundance levels remained unchanged, in Huh7 cells infected with HCV for 72 h (bottom panel). Interestingly, we observed that PRK2 levels also decreased at 17-DMAG concentrations greater than 250 nM. It remains to be characterized how PRK2 expression or stability is regulated by high concentrations of 17-DMAG. Nevertheless, this dose-related PRK2 abundance regulation by 17-DMAG might be attributed, at least in part, to more profound anti-HCV activity of 17-DMAG at higher concentrations (above 250 nM).

HCV IRES-mediated translation has been shown to be regulated by cell cycle [20]. We thus assessed by a dual reporter assay whether 17-DMAG regulates HCV IRES-mediated translation to rule out the possibility that anti-HCV activity of 17-DMAG might be caused by inhibition of viral protein expression. Huh7 cells were transfected with the dual reporter assay plasmid allowing cap-dependent expression of *Renilla* luciferase and HCV IRES-dependent translation of firefly luciferase, and treated with various concentrations of 17-DMAG for 72 h. The assay results showed that 17-DMAG did not affect HCV IRES-mediated translation (Fig. 4B). In addition, cell cycle was not significantly altered in Huh7 cells treated with 500 nM 17-DMAG, while LY294002, a PI3 kinase inhibitor used as a positive control for the assay, arrested cells at the G1-stage, as expected.

4. Discussion

In this study, first, we provide evidence that stabilization of PDK1 by Hsp90 is functionally linked to PRK2-mediated HCV NS5B phosphorylation. Our results revealed that inhibition of Hsp90 by 17-DMAG leads to destabilization of PDK1 through the proteosome-dependent pathway. PDK1 destabilization reduced the abundance of the active phosphorylated PRK2 level to negatively regulate HCV replication. Second, we show that the combination of 17-DMAG with HA1077, an inhibitor of PRK2 can effectively suppress HCV replication.

Previous studies demonstrated that an Hsp90/NS5A/FKBP8 complex plays a crucial role in HCV replication and that geldanamycin, an Hsp90 inhibitor, reduces the efficiency of HCV replication possibly by interfering with the binding of FKBP8 to Hsp90 and/or NS5A [9]. Recently, Ujino et al. [21] provided evidence that the Hsp90 inhibitor, 17-AAG, specifically destabilized HCV NS3 protein and that this destabilization was due to interaction between Hsp90 and the NS3 protein [9]. These findings and our results supports the notion that Hsp90 activity is functionally linked to HCV replication. Hsp90 expression levels are increased in cancer cells [22]. The enhanced Hsp90 expression is likely to result in stabilization of PDK1 and thereby activation of PRK2 to facilitate HCV replication. Supporting this possibility, our findings demonstrated that the anti-HCV activity of 17-DMAG indeed could be further enhanced when combined with the PRK2 inhibitor HA1077 or fasudil [23]. Thus, this combination may provide a promising alternative therapeutic option for IFN-nonresponsive HCV patients. Furthermore, 17-DMAG has been shown to have anticancer effects in clinical studies [18]; although, no controlled studies have yet been done in HCC patients. In addition, the Rho kinase inhibitory activity of HA1077 inhibited tumor cell motility and metastasis in human and rat tumor models [24]. Therefore, it would be of interest to test if 17-DMAG or in combination with HA1077 is also effective for the prevention of HCC progression along with its effects in suppressing HCV replication.

In conclusion, we provide a novel mechanism of how HCV replication is controlled by Hsp90 inhibition. Our results identified a critical role for Hsp90 in the regulation of HCV NS5B phosphorylation via the PDK1–PRK2 signaling pathway. Inhibitors targeting PDK1 or PRK2 or combinations of compounds targeting these kinases might be attractive therapeutic approaches for HCV.

Acknowledgments

This work was supported by a grant from the National Research Foundation of Korea (NRF-2011-0027650), and in part by the Technology Innovation Program (MKE 10035159) funded by the Ministry of Knowledge Economy (MKE, Korea) and by grants from National Research Foundation of Korea (NRF 2011-0001236 and 2010-0029116).

References

[1] D. Lavanchy, The global burden of hepatitis C, Liver Int. 29 (Suppl. 1) (2009) 74–81.
[2] G.R. Foster, Pegylated interferons for the treatment of chronic hepatitis C: pharmacological and clinical differences between peginterferon-alpha-2a and peginterferon-alpha-2b, Drugs 70 (2010) 147–165.
[3] R. Bartenschlager, V. Lohmann, Replication of hepatitis C virus, J. Gen. Virol. 81 (2000) 1631–1648.
[4] S.J. Kim, J.H. Kim, Y.G. Kim, H.S. Lim, J.W. Oh, Protein kinase C-related kinase 2 regulates hepatitis C virus RNA polymerase function by phosphorylation, J. Biol. Chem. 279 (2004) 50031–50041.
[5] S.J. Kim, J.H. Kim, J.M. Sun, M.G. Kim, J.W. Oh, Suppression of hepatitis C virus replication by protein kinase C-related kinase 2 inhibitors that block phosphorylation of viral RNA polymerase, J. Viral Hepat. 16 (2009) 697–704.
[6] D.B. Solit, G. Chiosis, Development and application of Hsp90 inhibitors, Drug Discov. Today 13 (2008) 38–43.
[7] R. Geller, M. Vignuzzi, R. Andino, J. Frydman, Evolutionary constraints on chaperone-mediated folding provide an antiviral approach refractory to development of drug resistance, Genes Dev. 21 (2007) 195–205.
[8] D.R. Smith, S. McCarthy, A. Chrovian, G. Olinger, A. Stossel, T.W. Geisbert, L.E. Hensley, J.H. Connor, Inhibition of heat-shock protein 90 reduces Ebola virus replication, Antiviral Res. 87 (2010) 187–194.
[9] T. Okamoto, Y. Nishimura, T. Ichimura, K. Suzuki, T. Miyamura, T. Suzuki, K. Moriishi, Y. Matsuura, Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90, EMBO J. 25 (2006) 5015–5025.

[10] S. Nakagawa, T. Umehara, C. Matsuda, S. Kuge, M. Sudoh, M. Kohara, Hsp90 inhibitors suppress HCV replication in replicon cells and humanized liver mice, Biochem. Biophys. Res. Commun. 353 (2007) 882–888.

[11] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, Nat. Med. 11 (2005) 791–796.

[12] J.M. Sun, S.J. Kim, G.W. Kim, J.K. Rhee, N.D. Kim, H. Jung, J. Jeun, S.H. Lee, S.H. Han, C.S. Shin, J.W. Oh, Inhibition of hepatitis C virus replication by Monascus pigment derivatives that interfere with viral RNA polymerase activity and the mevalonate biosynthesis pathway, J. Antimicrob. Chemother. 67 (2012) 49–58.

[13] D.G. Ahn, S.B. Shim, J.E. Moon, J.H. Kim, S.J. Kim, J.W. Oh, Interference of hepatitis C virus replication in cell culture by antisense peptide nucleic acids targeting the X-RNA, J. Viral Hepat. 18 (2011) e298–306.

[14] D.G. Ahn, W. Lee, J.K. Choi, S.J. Kim, E.P. Plant, F. Almazan, D.R. Taylor, L. Enjuanes, J.W. Oh, Interference of ribosomal frameshifting by antisense peptide nucleic acids suppresses SARS coronavirus replication, Antiviral Res. 91 (2011) 1–10.

[15] A.J. Collier, S. Tang, R.M. Elliott, Translation efficiencies of the 5′ untranslated region from representatives of the six major genotypes of hepatitis C virus using a novel bicistronic reporter assay system, J. Gen. Virol. 79 (1998) 2359–2366.

[16] N. Fujita, S. Sato, A. Ishida, T. Tsuruo, Involvement of Hsp90 in signaling and stability of 3-phosphoinositide-dependent kinase-1, J. Biol. Chem. 277 (2002) 10346–10353.

[17] A.D. Basso, D.B. Solit, P.N. Munster, N. Rosen, Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2, Oncogene 21 (2002) 1159–1166.

[18] S. Pacey, R.H. Wilson, M. Walton, M.M. Eatock, A. Hardcastle, A. Zetterlund, H.T. Arkenau, J. Moreno-Farre, U. Banerji, B. Roels, H. Peachey, W. Aherne, J.S. de Bono, F. Raynaud, P. Workman, I. Judson, A phase I study of the heat shock protein 90 inhibitor alvespimycin (17-DMAG) given intravenously to patients with advanced solid tumors, Clin. Cancer Res. 17 (2011) 1561–1570.

[19] A. Casamayor, N.A. Morrice, D.R. Alessi, Phosphorylation of Ser-241 is essential for the activity of 3-phosphoinositide-dependent protein kinase-1: identification of five sites of phosphorylation in vivo, Biochem. J. 342 (1999) 287–292.

[20] M. Honda, S. Kaneko, E. Matsushita, K. Kobayashi, G.A. Abell, S.M. Lemon, Cell cycle regulation of hepatitis C virus internal ribosomal entry site-directed translation, Gastroenterology 118 (2000) 152–162.

[21] S. Ujino, S. Yamaguchi, K. Shimotohno, H. Takaku, Heat-shock protein 90 is essential for stabilization of the hepatitis C virus nonstructural protein NS3, J. Biol. Chem. 284 (2009) 6841–6846.

[22] L. Neckers, S.P. Ivy, Heat shock protein 90, Curr. Opin. Oncol. 15 (2003) 419–424.

[23] S.P. Davies, H. Reddy, M. Caivano, P. Cohen, Specificity and mechanism of action of some commonly used protein kinase inhibitors, Biochem. J. 351 (2000) 95–105.

[24] L. Yin, K. Morishige, T. Takahashi, K. Hashimoto, S. Ogata, S. Tsutsumi, K. Takata, T. Ohta, J. Kawagoe, K. Takahashi, H. Kurachi, Fasudil inhibits vascular endothelial growth factor-induced angiogenesis in vitro and in vivo, Mol. Cancer Ther. 6 (2007) 1517–1525.