Chemistry & Biology 13, 1051–1060, October 2006 ©2006 Elsevier Ltd All rights reserved DOI 10.1016/j.chembiol.2006.08.010

A Small-Molecule Probe for Hepatitis C Virus Replication that Blocks Protein Folding

Bojana Rakic,[1,3] Jennifer Clarke,[1] Tammy-Lynn Tremblay,[2] Janet Taylor,[4] Karl Schreiber,[4] Ken M. Nelson,[4] Suzanne R. Abrams,[4] and John Paul Pezacki[1,3,*]

[1]The Steacie Institute for Molecular Sciences
[2]Institute for Biological Sciences
National Research Council Canada
100 Sussex Drive
Ottawa, Ontario K1A 0R6
Canada
[3]Department of Chemistry
University of Ottawa
10 Marie-Curie
Ottawa, Ontario K1N 6N5
Canada
[4]Plant Biotechnology Institute
National Research Council Canada
110 Gymnasium Place
Saskatoon, Saskatchewan S7N 0W9
Canada

Summary

The hepatitis C virus (HCV) is a growing global health problem. Small molecules that interfere with host-viral interactions can serve as powerful tools for elucidating the molecular mechanisms of pathogenesis and defining new strategies for therapeutic development. Using a cell-based screen involving subgenomic HCV replicons, we identified the ability of 18 different abscisic acid (ABA) analogs, originally developed as plant growth regulators, to inhibit HCV replication. Three of these were further studied. One compound, here named origamicin, showed antiviral activity through the inhibition of host proteins involved in protein folding. Origamicin could therefore be an important tool for studying the maturation of both host and viral proteins. Herein we demonstrate an application for molecular scaffolds based on ABA for mammalian cell targets involved in protein folding.

Introduction

Affecting over 170 million individuals worldwide, the hepatitis C virus (HCV) is a growing threat to global health [1]. No vaccine currently exists, and the availability of clinical treatments is limited [2–8]. HCV is a positive-strand RNA virus of the Flaviviridae family and has an ~9.6 kb genome (Figure 1), which encodes for an ~3000 amino acid polyprotein that is posttranslationally cleaved into functional components [9, 10]. The HCV genome encodes for both structural proteins, which make up the virion particles (the core protein and envelope proteins, E1 and E2), and nonstructural proteins (NS2–NS5B). The NS3 protein is a protease that, in conjunction with NS4A, mediates all cleavages in the NS3–NS5B region [9, 10]. NS3 also has nucleoside triphosphatase and helicase activities. NS5A is a highly phosphorylated protein that is thought to be involved in the resistance to interferon α [9, 10]. NS5B is an RNA-dependent RNA polymerase. Given the global health issue presented by HCV, there is great interest in elucidating the role of host-virus protein interactions in propagation. The functional collaboration between host and virus proteins that is required for HCV to replicate defines the molecular basis of pathogenesis, and it represents a suitable target for drug development [11]. In fact, HCV relies on host as well as viral proteins for posttranslational modifications of the initially translated HCV polyprotein. These modifications are essential for maturation of the structural and nonstructural proteins. For example, a host cell ER signal peptidase cleaves the HCV polyprotein to yield immature structural proteins that are then glycosylated by other host cell proteins [10]. The glycosylation of the HCV E1 and E2 envelope proteins by host glycosidases is of particular importance to HCV virulence because it is required for viral particle formation and budding, efficient host cell recognition, and viral entry [12, 13].

High-throughput techniques including gene expression profiling [14, 15] and proteomics approaches have identified a number of important host-virus interactions in cell culture models for HCV replication and in HCV infection [16–20]. Small-molecule-based antiviral strategies have also demonstrated success in inhibiting HCV in clinical infections [21]. There is much promise in the use of chemical genetic approaches [22, 23] to discover new host-pathogen interactions because many dynamic molecular interactions are not easily identified. Chemical genetics approaches have uncovered cell-permeable small molecules that disrupt protein function in cellular processes such as mitosis [24], cell signaling [25], and tissue regeneration [26]. Here, we have combined small-molecule screening with activity-based inhibitor profiling [27] to identify natural product-like inhibitors of HCV replication and to determine the target proteins to which these inhibitors bind. Our small-molecule library is based on the plant hormone (+)-S-abscisic acid (ABA), which is a carotenoid-derived sesquiterpene. ABA is involved in the regulation of numerous physiological processes in plant growth and development, including embryo and seed development, protein storage, and stress responses [28]. Many analogs of ABA have been synthesized for structure-activity studies and to develop growth regulators with enhanced and prolonged activity [29–33]. ABA also has a high degree of structural similarity to terpenoid ligands such as retinoic acid, as well as synthetic mammalian nuclear hormone receptor ligands [34, 35]. Since we recently found that antagonists of peroxisome proliferator-activated receptor (PPAR) block HCV replication [36], we sought to determine if any ABA analogs previously developed to identify biologically active plant growth regulators would show any anti-HCV activity.

Results and Discussion

In order to determine whether analogs with the ABA scaffold demonstrate activity against HCV, a 100 compound

*Correspondence: john.pezacki@nrc-cnrc.gc.ca

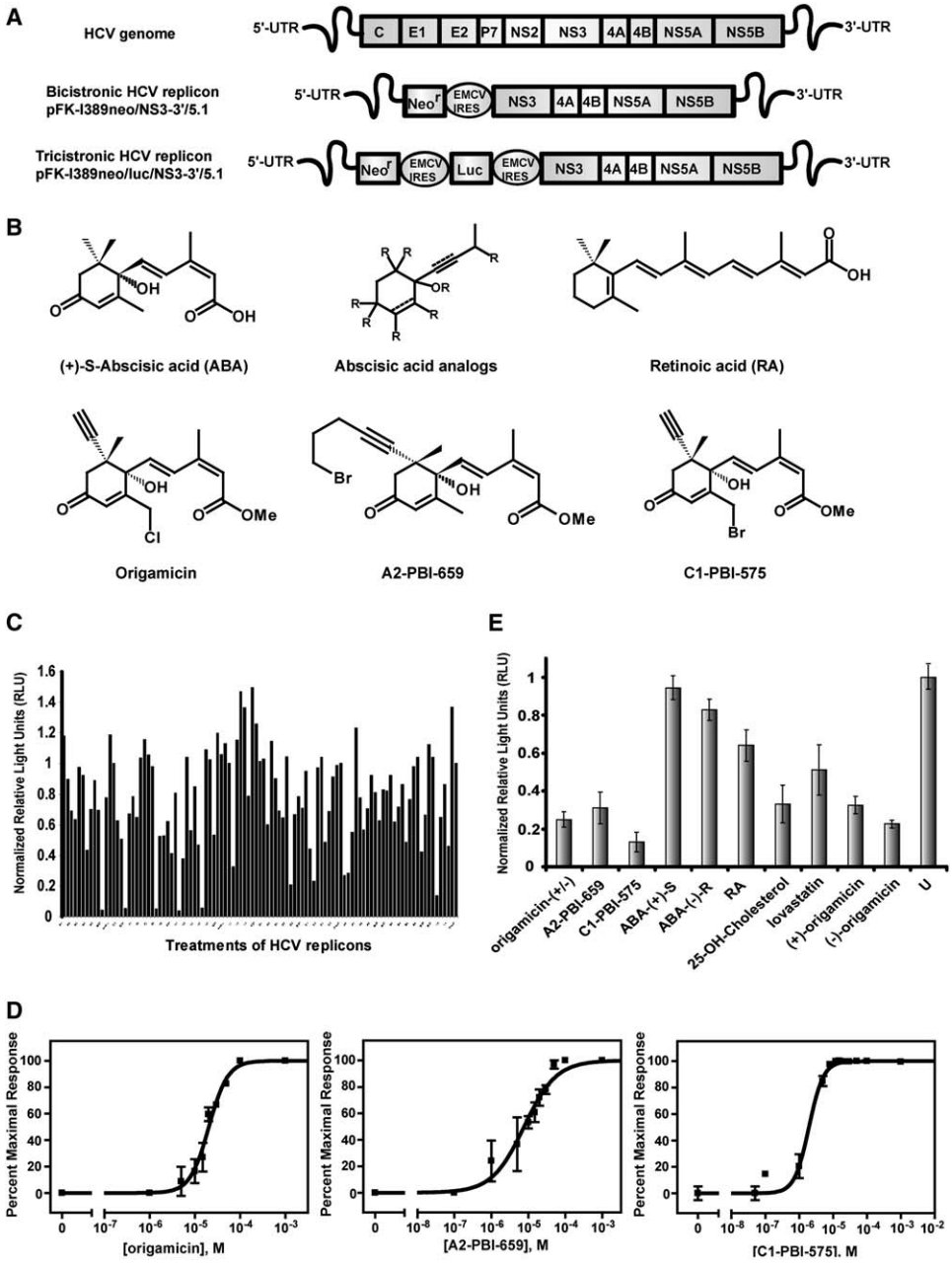

Figure 1. Activity of ABA Analogs in HCV Replicon-Bearing Huh-7 Human Hepatoma Cells

(A) Schematic representations of the HCV genome and the HCV replicons used in this study.

(B) Chemical structures.

(C) Luciferase reporter gene activity of HCV replicons expressing the luciferase gene. Activities were determined at 100 μM concentration after 24 hr treatments.

(D) Percent maximum signal versus concentration plots from which $IC_{50}^{Replicon}$ values were determined for origamicin, A2-PBI-659, and C1-PBI-575 after a 24 hr treatment.

(E) Luciferase reporter gene activity of HCV replicons expressing the luciferase gene determined after a 24 hr treatment for the following compounds: (±) origamicin at 30 μM, A2-PBI-659 at 20 μM, C1-PBI-575 at 5 μM; (+)-S-abscisic acid (ABA) and (−)-R-abscisic acid (ABA) at 100 μM; retinoic acid (RA) at 100 μM; lovastatin at 50 μM; 25-hydroxycholesterol at 2.5 μM; (+) origamicin at 30 μM; (−) origamicin at 30 μM. "U" denotes untreated cells.

Error bars represent the mean ± SD.

library of ABA analogs was screened with a tricistronic subgenomic HCV replicon containing the genetic reporter firefly luciferase (Figure 1A) [36, 37]. All of the ABA analogs contained the six-membered ring and the unsaturated side chain with a variety of substituents (Figure 1B). For the initial screening, a concentration of 100 μM of the compounds was used. After a 24 hr treatment, the Huh-7 cells harboring the tricistronic HCV replicon were lysed, and the amount of HCV replication was measured by using luciferase assays [36, 37]. Initial

screening identified 18 ABA analogs that showed significant inhibitory activity in the genetic reporter assay for HCV replication (Figure 1C). These analogs were then tested for cytotoxicity at the same concentration, 100 μM, by quantifying lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH in culture supernatants is measured with a 30 min coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazan product. The amount of color formed is proportional to the number of lysed cells and is a direct measurement of cell viability (see the Supplemental Data available with this article online). Experiments showed that all of the 18 compounds identified as having anti-HCV activity in the luciferase assay displayed some degree of cytotoxicity at a 100 μM concentration, with cell viabilities ranging from 9.4% to 70% compared to untreated cells (see Figure S1 in the Supplemental Data). To determine whether any of the compounds showed activity at lower concentrations, we repeated the assay between 100 μM and 50 nM concentrations (data not shown), and the firefly luciferase and cytotoxicity assays of these compounds showed that origamicin, A2-PBI-659, and C1-PBI-575 had the greatest antiviral activity and only C1-PBI-575 showed adverse cytotoxicity at concentrations less than 100 μM (Figure S2).

We evaluated the efficacy of the three ABA analogs toward HCV inhibition by using the tricistronic HCV replicon (Figure 1A) and by measuring firefly luciferase activity over a wide range of concentrations in order to determine the $IC_{50}^{Replicon}$ values (Figure 1D). These values were determined to be 20.5 ± 2.5 μM, 10.6 ± 6.5 μM, and 1.98 ± 0.42 μM for origamicin, A2-PBI-659, and C1-PBI-575, respectively. At concentrations at which cell viability was comparable, these analogs showed similar or superior inhibitory effects compared to the known HCV inhibitors 25-hydroxycholesterol [15, 37, 38] (2.5 μM) and lovastatin [38] (50 μM) (Figure 1E). Treatment of the HCV replicon with 100 μM of (+)-S-abscisic acid and (−)-R-abscisic acid did not affect HCV replication (Figure 1E). The three above-mentioned ABA analogs showed similar cytotoxicity to that of retinoic acid, a mammalian ortholog of ABA, at comparable concentrations (Figure S4). However, retinoic acid showed much less activity against the HCV replicon compared to the other small molecules (Figure 1E). Experiments with the R and S isomers of origamicin and HCV replicons were conducted at several concentrations of ABA analog, and the results indicated that the antiviral properties toward the HCV replicon are not dependent on stereochemistry. The position of the halogen in origamicin was critical for its biological activity (data not shown). For example, replacing the allylic halide in origamicin or C1-PBI-575 with a propyl halide eliminated the biological activity. The cytotoxicity of halogenated electrophiles toward human liver cells has been established previously [39].

In order to validate the luciferase assay data, the effects of ABA analogs origamicin, A2-PBI-659, and C1-PBI-575 on expression levels of HCV proteins were measured by using a different bicistronic HCV replicon (Figure 1A). Huh-7 cells stably supporting HCV replicon propagation were treated with ABA analogs for 12–30 hr, after which the cells were lysed, the cellular proteins were collected, and quantified western blots were performed and probed against HCV NS3 and NS5A proteins by using mouse anti-NS3 and anti-NS5A primary antibodies (Figure 2A). Antibodies against the PTP1D protein showed that the concentrations used were not toxic for the cells and that there was equal loading of proteins on the blots. The western blots showed that compounds origamicin, A2-PBI-659, and C1-PBI-575 inhibited HCV NS5A protein production by ~80%, 75%, and greater than 90%, respectively, which was comparable to the inhibition exhibited by antiviral cytokine interferon γ (IFNγ) (Figure 2A). Interestingly, after longer treatment with ABA analogs origamicin and A2-PBI-659, the replicon cells recovered and HCV replication resumed. In contrast, cells could be cured of HCV replicons by using the ABA analog C1-PBI-575.

In order to determine if the inhibition of HCV replication was linked with changes in cellular viability based on the cell number or morphology, we performed fluorescence microscopy experiments on the tricistronic HCV replicon treated with origamicin, A2-PBI-659, and C1-PBI-575 at a variety of concentrations (Figure 2B). Cells were treated for 48 hr, after which the nuclei were stained with a mouse anti-NS5B primary antibody and a fluorescein-labeled anti-mouse secondary antibody, followed by counterstaining with DAPI to label the nuclei and Oil-Red O to visualize the lipid droplets. We observed decreases in NS5B protein with origamicin at 20 μM, with A2-PBI-659 at 20 μM, and with C1-PBI-575 at 7 μM. However, cell viability, lipid droplet content, and cell morphology were identical to those of the untreated cells. Fluorescence microscopy showed that at these concentrations there was no change in cellular viability, as shown by nuclear morphology, based on DAPI; however, some cytotoxicity was observed according to the LDH assay (Figure S3).

To investigate if HCV inhibition by the active ABA analogs was due to interactions with the host Huh-7 human hepatoma proteins, or whether their activity and cytotoxicity were due to a specific interaction with HCV, their cytotoxic effects were screened against a panel of human cell lines. These cell lines included Huh-7, HepG2 and C3A human hepatocarcinoma, MDA-MB-435 human melanoma, HEK293 human epithelial kidney, and HeLa cervical carcinoma cells (Figure S3). The results suggested that the host cell effects of origamicin, A2-PBI-659, and C1-PBI-575 are cell-type specific. Hepatoma and kidney cells appeared to be most sensitive to the effects of the ABA analogs, whereas HeLa cells showed virtually no cytotoxicity upon treatment with the ABA analogs, excepting high doses of C1-PBI-575 (≥100 μM). Therefore, we conclude that the cytotoxic effect at higher concentrations of ABA analogs is dependent on cell type but does not require the expression of HCV proteins.

As ABA and its analogs have similar molecular structures to retinoic acid and other nuclear hormone receptor ligands, they may show reactivity toward these mammalian receptors. In order to test whether the ABA analogs affect the RXR pathway, we performed the albumin ELISA assay with analogs origamicin, A2-PBI-659, C1-PBI-575, as well as lovastatin, 25-hydroxycholesterol, and ABA, and we compared the results to those with retinoic acid and untreated cells (Figure 2C). We

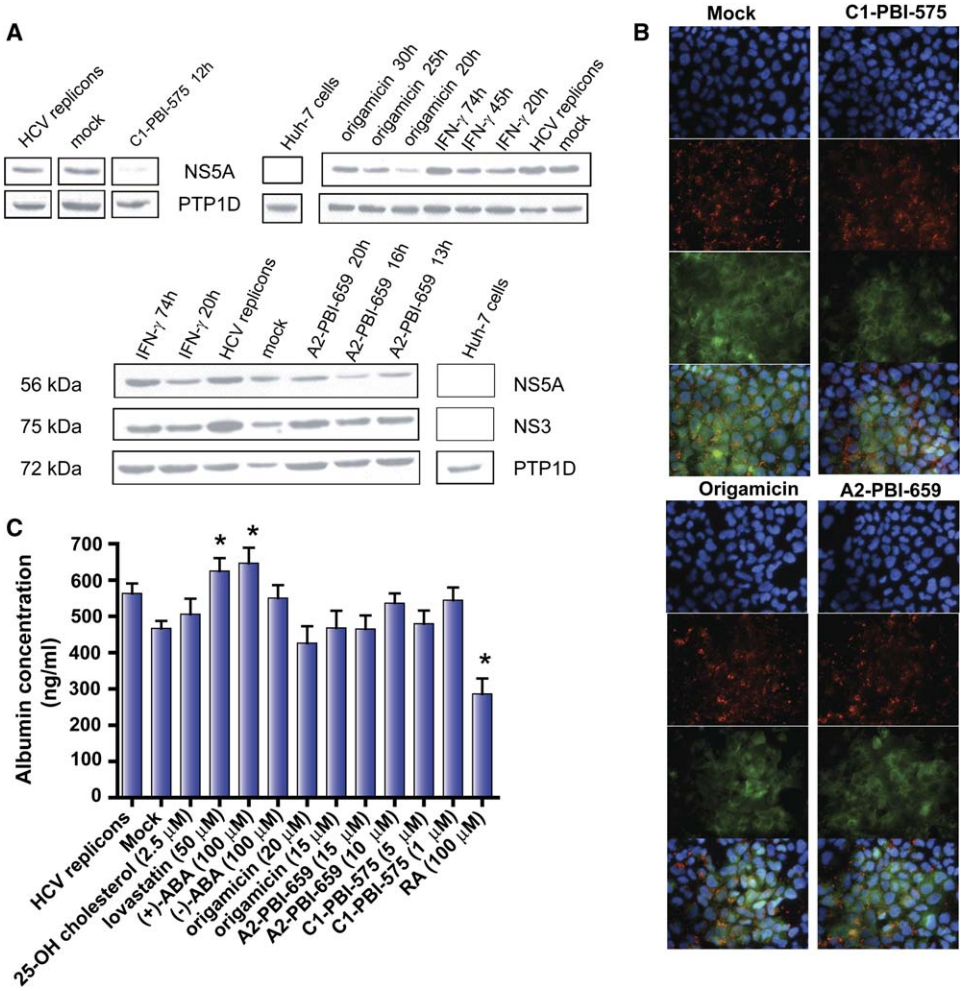

Figure 2. Functional Validation of ABA Analog Activity in HCV Replicon-Bearing Huh-7 Human Hepatoma Cell Lines

(A) Western blots of HCV NS5A and NS3 proteins from subgenomic HCV replicons under various conditions. Lanes for C1-PBI-574 (upper left) are: HCV replicon cells, untreated; mock treatment with 1% v/v MeOH; C1-PBI-575, 15 μM for a 12 hr treatment. Lanes for origamicin (upper right) are: Huh-7 cells, untreated; origamicin, 50 μM for 30 hr, 25 hr, and 20 hr treatments, respectively; IFNγ, 5 U/ml at 74 hr, 45 hr, and 20 hr treatments, respectively; HCV replicons, untreated; and mock treatment with 1% v/v MeOH. Lanes for A2-PBI-569 (lower middle) are: IFNγ, 5U/ml at 74 hr and 20 hr treatments, respectively; HCV replicons, untreated; mock treatment with 1% v/v MeOH; A2-PBI-569, 50 μM at 20 hr, 16 hr, and 13 hr treatments, respectively; Huh-7 cells, negative control. Blots were also probed for PTP1D expression to indicate the loading consistency.

(B) Fluorescence microscopy of immunostained HCV replicon cells. Panels from top to bottom are: cell nuclei stained blue with 4′,6-diamino-2-phenylindole (DAPI), lipid droplets stained red with Oil Red O, HCV NS5B protein stained green, and the three-color overlay. "Mock" is treatment with 1% v/v MeOH, the C1-PBI-575 analog is treated at 7 μM for 48 hr, the origamicin analog is treated at 20 μM for 48 hr, and the A2-PBI-569 analog is treated at 20 μM for 48 hr.

(C) Secreted albumin (ng/ml) from HCV replicon-bearing cells as determined after a 24 hr treatment with the indicated compounds. Analog concentrations are given in parentheses. Error bars represent the mean ± SD, and asterisks indicate a significant difference ($p < 0.05$) relative to the untreated HCV replicons.

chose to investigate albumin levels because their secretion is known to be directly modulated by retinoic acid. Also, changes in albumin levels can be associated with other effects on cell proliferation and differentiation by ABA and its analogs [35, 40, 41]. Huh-7 cells stably expressing HCV replicons were treated with the analogs for 24 hr, after which albumin ELISA assays were performed. No significant reduction in albumin was observed upon treatment with ABA or the three analogs that showed anti-HCV activity, in contrast to retinoic acid, which did lower the secreted albumin levels as expected.

To understand the possible mechanisms by which the ABA analogs give rise to antiviral activity against HCV, we utilized a chemical proteomics strategy to determine the protein targets within the entire proteome [27]. We applied inhibitor-based protein-profiling approaches to investigate interactions of the ABA analog origamicin on both host and viral proteomes isolated from the replicon systems. We exploited the alkyne functionality present in origamicin to conjugate both rhodamine for in-gel fluorescence experiments [27] and biotin for affinity chromatography experiments by using "click" chemistry [42] (Figure 3A).

Our first experiments to identify the ABA targets involved in vitro labeling of cytosolic and membrane proteins from Huh-7 cells harboring HCV replicons by

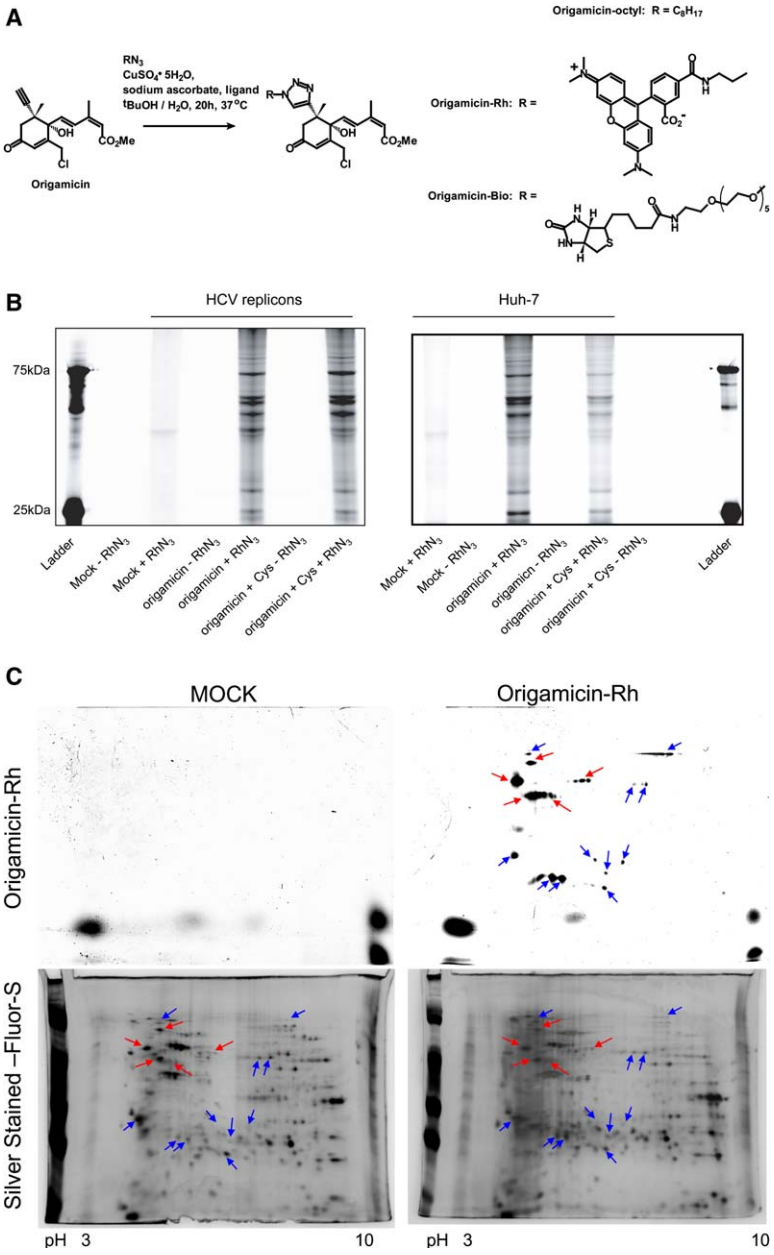

Figure 3. Proteomics Analyses of Origamicin Activity in HCV Replicon Cells and Huh-7 Parental Cells

(A) Scheme for azide-alkyne "click" coupling reactions between origamicin and various alkyl azides.

(B) In-gel fluorescence images of SDS-PAGE gels containing labeled proteomes from origamicin-treated cells, where origamicin was preincubated with cells for 24 hr at 30 μM concentration, followed by proteome extraction and a "click" reaction of the isolated proteome with RhN_3. Arrows indicate four strongly labeled proteins.

(C) Fluorescent and silver-stained two-dimensional gel images of untreated control (Mock) and treated (origamicin) Huh-7 cytosolic proteins. Treated cells were preincubated for 24 hr with 30 μM origamicin, followed by protein extraction and a "click" reaction of the isolated proteins with RhN_3. The mock gel was done with proteins extracted from untreated cells that were submitted to the same "click" reaction conditions. Arrows are pointing to major spots that were chosen for protein identification by LC-MS/MS. Red arrows are pointing to spots containing protein of the PDI family (PDIA1, PDIA3, PDIA4, and PDIA6).

origamicin-Rh, followed by isolation of proteins with origamicin-Bio. Four strong bands were observed upon incubation of the biotin-labeled probe origamicin-Bio (Figure 3A) with cytosolic proteins that were purified and identified via mass spectrometry (data not shown). Interestingly, the four proteins were all host proteins important for protein folding and trafficking, specifically heat shock proteins and protein disulfide isomerase. We postulated that origamicin reacted with these targets as an electrophile. The electophilic sites can be blocked with cysteine, which can potentially reduce the activity of origamicin. Luciferase assays involving the HCV replicon indicated that preincubation of origamicin at 30 μM with cysteine significantly reduced origamicin activity, whereas preincubation of origamicin at 50 μM with cysteine only partially affected the activity of origamicin, which can be attributed to reversible interconversion between origamicin and its cysteine adduct. Statistical analysis showed that incubation with cysteine significantly reduced the activity and toxicity of origamicin ($p < 0.05$); however, some activity was retained and cell viability did not completely recover in the presence of cysteine, indicating that cysteine alone was insufficient to irreversibly block origamicin's inhibitory activity (Figure S5). Several examples exist whereby small-molecule inhibitors are modified prior to their interaction with their target protein. For example, lactacystin is known to undergo reversible interconversion between a lactone and thioacyl intermediates [43–45].

In order to more specifically characterize the protein targets of the ABA analog origamicin, in situ inhibitor-based protein profiling was conducted. HCV replicon-bearing cells were treated for 24 hr with the analog origamicin and origamicin preincubated with cysteine. The cytosolic proteins were then extracted, and copper-catalyzed click reactions were conducted to

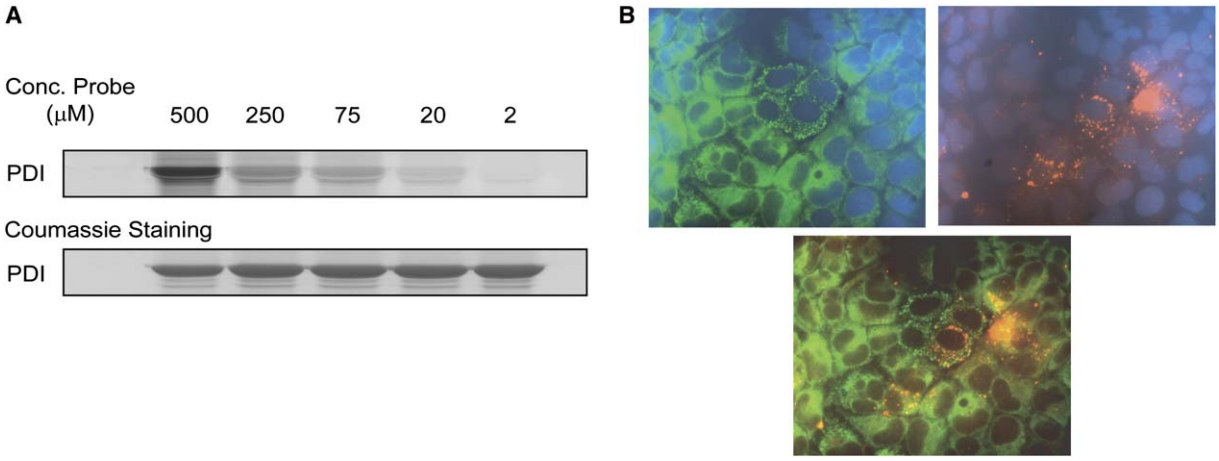

Figure 4. Validation of Origamicin Activity toward Protein Disulfide Isomerase Proteins

(A) An in-gel fluorescence image of an SDS-PAGE gel containing pure yeast PDI incubated with various concentrations of origamicin-Rh (upper panel) and Coomassie blue staining of the gel (lower panel).

(B) Fluorescence microscopy images of HCV replicon-bearing cells that were treated with origamicin-Rh (red, upper right) and counterstained with antibodies against PDI (green, upper left). Nuclei (blue) are stained with 4′,6-diamino-2-phenylindole (DAPI). The overlay in the lower panel shows that nearly all origamicin-Rh is localized in the ER and colocalized with PDI.

conjugate rhodamine to the proteins bound to the ABA analogs. In-gel fluorescence again showed that origamicin interacted strongly with four proteins (Figure 3B). In order to confirm the specificity and to determine other possible protein targets for origamicin, we performed two-dimensional gel electrophoresis experiments (Figure 3C). We observed 8 strongly fluorescent proteins and 16 weakly labeled proteins. Proteins that were identified by nanoLC-MS/MS either had a cysteine-containing active site (e.g., protein disulfide isomerase family, and, to a lesser extent, peroxiredoxin 1) or were known phosphoproteins (e.g., heat-shock protein β-1, cofilin-1, and nucleophosmin). The strongest fluorescence, and thus the highest selectivity in labeling, was observed with spots containing proteins of the PDI family (PDIA1, PDIA3, PDIA4, and PDIA6), which all have two cysteine-containing active sites. The targeting of PDI proteins by origamicin is consistent with the reactivity of other simpler halogenated organic molecules [39, 46, 47] that demonstrate a lower degree of target specificity and a much higher degree of cytotoxicity [39, 46, 47]. The full list of protein identities, protocols, and methods is provided in Supplemental Data. One of the cysteine-containing active site proteins that was identified is retinal dehydrogenase 1. This is consistent with our original hypothesis that ABA analogs may show crossreactivity to retinoic acid-binding proteins.

Since origamicin was found to interact strongly with protein disulfide isomerases, we examined its interaction with yeast PDI in vitro. We found that the interaction of origamicin-Rh was concentration dependent and had a micromolar binding constant (Figure 4A). We also examined whether origamicin showed subcellular localization to the endoplasmic reticulum (ER), where protein synthesis and folding occurs. We performed fluorescence microscopy on HCV replicon cells treated with origamicin-Rh and counterstained with antibodies against PDI. Origamicin-Rh did localize to the ER and colocalized with PDI (Figure 4B).

To demonstrate whether origamicin inhibits PDI activity, we conducted activity assays in *Escherichia coli* strains JCB 570 and JCB 571, which differ only by the disruption of the *dsbA* (disulfide bond A) gene. Knockout strains have a severely impaired capacity for disulfide bond formation, resulting in a range of defects, including significantly reduced activity of the enzyme alkaline phosphatase (AP) [48]. The mutant phenotype can be reversed by heterologous expression of protein disulfide isomerases [49]. This complementation can be attributed directly to the restoration of enzymatically catalyzed disulfide bond formation. The *E. coli* strain JCB 571 has a transposon insertion in the *dsbA* gene, thus eliminating endogenous disulfide oxidase activity. An expression vector containing the *Brassica carinata PDI1* gene that contains a highly homologous active site to that of human PDIs was previously introduced into this mutant and was used to demonstrate PDI activity in the encoded protein (unpublished data). We conducted PDI inhibition assays by using cultures of the JCB 571 transformant and a wild-type strain (JCB 570). These were incubated with origamicin as well as ABA as a control prior to evaluating PDI activity via a colorimetric assay of AP activity [50, 51]. The AP activity in the wild-type strain was unaffected by concentrations of origamicin ranging from 0 to 1 mM (Figure 5). However, a dose-dependent decrease in AP activity was observed in the *dsbA* mutant expressing a *Brassica carinata* PDI (Figure 5). These observations show that origamicin targets eukaryotic PDIs and is able to distinguish between *B. carinata* PDI and the bacterial DsbA.

The *E. coli* DsbA is a 21 kDa soluble protein composed of a single thioredoxin fold and a CXXC active site motif. It is a strong catalyst of protein/peptide cysteine oxidation and has some isomerase activity. However, it is DsbC, as a dimeric enzyme, that is primarily responsible for the isomerization of incorrect disulfide bonds. DsbC is a 25 kDa protein and, similarly to DsbA, has a single thioredoxin fold and active site motif. In contrast, human

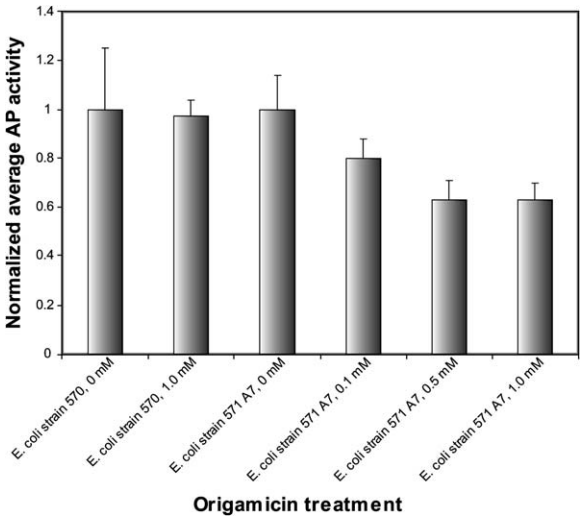

Figure 5. Evaluation of Origamicin Dosage Effects in a Protein Disulfide Isomerase Inhibition Assay

Cultures of *Escherichia coli* (570 = wild-type; 571 A7 = *dsbA* mutant expressing a *Brassica carinata* PDI) were incubated with 0, 0.1, 0.5, or 1.0 mM origamicin for 4 hr at 37°C. The activity of PDI was subsequently assessed by measuring alkaline phosphatase (AP) activity. Error bars represent the standard deviation between measurements.

and plant PDI are ~55 kDa proteins, each with two thioredoxin folds and two active site motifs. These enzymes catalyze both cysteine oxidation and disulfide bond isomerization [52, 53]. Furthermore, a study of complementation of *dsbA* and *dsbC* mutants with mutagenized human PDI indicated that residues in both active site motifs of the human PDI were involved in restoring AP activity [54]. Therefore, it is likely that the structural differences are responsible for the difference in response to origamicin between prokaryotic and eukaryotic enzymes.

It is known that a number of HCV proteins also colocalize with PDI in the ER. Therefore, small-molecule perturbation of PDI and the resultant induction of ER stress [55–61] could affect the proper maturation of HCV proteins and disrupt the environment of HCV replication complexes. Also, the disruption of the HIV lifecycle by blocking PDI function has been demonstrated by using the antimicrobial peptide bacitracin and anti-PDI antibodies [55, 56, 60, 61]. Protein disulfide isomerase function is required for the reduction of two disulfide bonds of HIV envelope glycoprotein 120 [55, 60, 61]. Inhibitors of PDI block cleavage of disulfide bonds in receptor-bound glycoprotein 120 and prevent HIV-1 entry [55]. Small molecules that target the ER have great potential as tools for cell biology because of the numerous fundamental processes that occur on the ER membrane. This is particularly true for HCV, for many other RNA viruses that replicate on the ER, and for those that require the protein-folding machinery for proper virion particle assembly [55–61]. The subcellular localization of origamicin is most likely linked to the high degree of specificity that it displays toward isoforms of PDI that reside in the ER. This feature helps establish it as a tool for understanding PDI function.

Origamicin's main protein targets in Huh-7 cells are protein disulfide isomerases. Dysfunction of these proteins can lead to ER stress due to the misfolding of both host and virus proteins, which, in turn, leads to unfolded protein response. It is known that HCV gene expression occurs on the ER and in itself induces ER stress. HCV also blocks the normal unfolded protein response and the resultant increase in protein degradation by suppressing the IRE1-XBP1 pathway, demonstrated in HCV replicons [62]. Cellular protein-folding machinery is necessary for the maturation of the HCV proteome. There are a number of disulfide bonds that are critical to HCV proteins, including the envelope proteins E1 and E2 as well as the NS5A protein [63–65]. PDI has also been shown to be important in maintaining the HIV envelope protein (Env) through reduction of the gp120 glycoprotein component [55–61]. The HCV NS3 serine protease and NS4A cofactor both colocalize with PDI, suggesting that their assembly and function are linked to these ER proteins. Therefore, we have proposed a model for the mechanism by which origamicin inhibits HCV replication (Figure 6). Although it is possible that other mechanisms are responsible for the antiviral

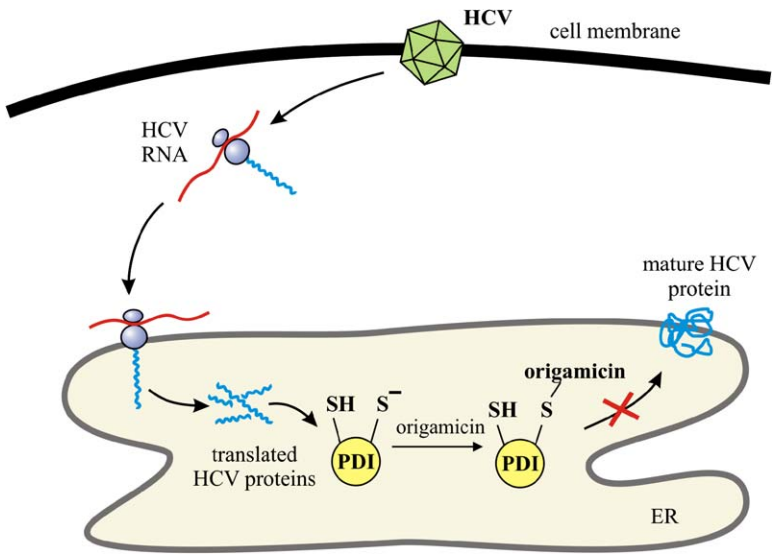

Figure 6. A Proposed Scheme for How Origamicin Inhibits HCV Replication

activity of origamicin, our hypothesis is that HCV replicon RNA can be translated by ribosomes into polyproteins and can be posttranslationally processed into immature proteins, but that origamicin prevents the proper folding of the precursor proteins, thus preventing the formation of mature viral proteins.

Significance

We have used chemical genetics approaches to establish a cell-permeable small-molecule modulator of protein folding that also acts as an inhibitor of HCV replication by blocking a critical host cell pathway that we believe is essential to the viral lifecycle. The protein targets of origamicin were determined by using inhibitor-based protein-profiling techniques that utilized in vivo reactions of origamicin with the HCV replicon cells, followed by a "click" reaction of the extracted proteins with either rhodamine or biotin labels. The major target for origamicin was found to be protein disulfide isomerase, a major component of the ER. To our knowledge, we herein establish a new way of targeting these specific ER proteins. We demonstrate that transient blocking of this host protein can block HCV replication in HCV replicons without permanently influencing cell viability, and we establish a strategy for anti-HCV compound development. Since origamicin is a close structural analog of a naturally occurring plant metabolite, originally developed as a plant growth regulator, its potency and specificity toward human proteins is surprising. These results demonstrate the opportunities for utilizing molecular scaffolds for multiple unrelated targets. There are many examples of plant metabolites with significant biological activity, and origamicin is another important example.

Experimental Procedures

Cell Culture and HCV Replicon Treatments

Cell monolayers of the human hepatoma cell line Huh-7 were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Burlington, ON) supplemented with 100 nM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum (FBS) (Cansera, Rexdale, ON). G418 was added to a concentration of 250 μg/ml to the Huh-7 cells stably expressing HCV replicons. The pFK-I389neo/NS3-3′/5.1 and pFK-I389neo/luc/NS3-3′/5.1 plasmids that contain HCV subgenomic replicons were utilized as previously described [36, 37]. For detailed protocols, see Supplemental Data.

LDH-Cytotoxicity Assay

Huh-7 cells harboring a subgenomic replicon were treated with small molecules as described above. Cells were washed and lysed with Triton X-100, and the cytotoxicity was determined by using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Maddison, WI) according to the manufacturer's protocol. The absorbance was read at 490/650 nm by using a Spectramax M2 plate reader (Molecular Devices Corporation, Sunnyvale, CA).

Fluorescence Microscopy

Fluorescent imaging of Huh-7 cells stably expressing HCV replicons was done by using an Axiovert 200M inverted microscope from Zeiss (North York, ON) with the Axiovision 3.1 software and an AxioCam connected to the microscope. For imaging of HCV proteins, cells were washed and fixed with 3.7% formaldehyde for 30 min at room temperature. Cells were probed with mouse anti-NS5B (5B-12B7) primary antibody (1:100 dilution; kindly provided by Darius Moradpour, Department of Medicine, University of Freiburg, Germany), followed by incubation with Cy2-conjugated donkey anti-mouse IgG secondary antibody (1:100 dilution; Jackson ImmunoResearch Laboratories Inc., Westgrove, PA). Nuclei were stained with DAPI (300 nM in PBS; Molecular Probes, Eugene, OR), and lipids were stained with Oil Red O (0.5% in isopropanol; Sigma-Aldrich, Oakville, ON). For imaging of the endoplasmic reticulum, cells at 70%–80% confluency were treated with 30 μM origamicin-Rh for 30 min. Cells were fixed and stained by using the Select FX Alexa Fluor 488 Endoplasmic Reticulum Labeling Kit (Molecular Probes) according to the manufacturer's protocol. Briefly, cells were washed with warm 1× PBS and fixed with 1× fixative solution at 37°C for 15 min. Cells were then permeabilized for 5 min. For ER staining, cells were stained with anti-PDI primary antibody (1:1000) dilution for 2 hr at 25°C, followed by incubation with the Alexa Fluor 488 secondary antibody at 25°C for 30 min.

Western Blotting

Huh-7 cells harboring subgenomic replicons (1×10^6 cells) were seeded in 60 mm dishes for preparation of western blot lysates. At 80% confluency, the cells were treated with small molecules. Cells were washed and lysed with an SDS lysis buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerol. SDS-PAGE gels and immunoblotting were then conducted as described previously [36, 37].

Albumin ELISA

Albumin ELISA assay was performed by using the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories, Inc., Montgomery, TX) and by following the manufacturer's protocol. Readings were taken at 450 nm by using a Spectramax M2 plate reader. See Supplemental Data.

Inhibitor-Based Protein Profiling

The proteins of HCV replicon-bearing Huh-7 cells as well as the parental cell line were probed under a variety of conditions by using approaches typically applied to activity-based protein profiling [27]. The description of the biotin and rhodamine linkers, ABA analog conjugates, and detailed protocols for the proteomics studies performed herein are available in Supplemental Data.

Analysis of PDI Activity in the Presence of Origamicin

Assessments of PDI inhibition by ABA analogs were performed in *Escherichia coli* strains JCB 570 (*ara*D139 Δ[*ara-leu*]7697 *gal*U *gal*K Δ*lac*X74 *rps*L *thi pho*R zih12::Tn10) and JCB 571 [48, 49], which differ only by the disruption of the *dsbA* gene (*dsbA*::kan1). Both strains were provided by J. Beckwith (Harvard Medical School, Department of Microbiology and Molecular Genetics, Boston, MA). For PDI inhibition assays, cultures of the JCB 571 transformant and a wild-type strain (JCB 570) were incubated with analogs of ABA prior to evaluating PDI activity via a colorimetric assay of AP activity [50]. Specifically, bacterial strains were cultured overnight, with shaking, at 37°C in "121 peptone" medium [51]. For strain JCB 570, this medium was supplemented with 12 μg/ml tetracycline, while media for cultures of JCB 571 transformants included both 25 μg/ml kanamycin and 50 μg/ml ampicillin. A total of 30 μl of each overnight culture was added to 3 ml of the same media (as two replicate subcultures). In addition, ABA and origamicin were added to separate subcultures to a final concentration of either 0.1 or 0.5 mM. (The compounds were dissolved in DMSO for 100 mM stock solutions; thus, control cultures contained either 0.1% or 0.5% DMSO.) Incubation continued for another 4 hr, after which 1 ml of each subculture was withdrawn and centrifuged at 6000 × g, and the supernatant was removed by aspiration. The bacterial pellet was resuspended in 1 ml of 1 M Tris-HCl (pH 8.0) and was transferred to a disposable cuvette (VWR). Two samples were prepared from each subculture (i.e., two replicates). The optical density of each sample when exposed to light at wavelengths of 420 and 600 nm was recorded in a Spectronic Unicam Genesys 10UV spectrophotometer (Rochester, NY). The assay of AP activity was initiated by the addition of 100 μl Sigma 104 solution (0.4 % [w/v] in 1M Tris-HCl [pH 8.0]) to each sample. These were incubated at 37°C until a yellow color began to develop in the positive control sample (strain JCB 570). The assay reaction was then stopped in one replicate of samples by adding 100 μl of 1 M K_2HPO_4. Another measurement

of optical density at 420 nm was performed on these samples. The remaining replicate was analyzed in a likewise manner at an arbitrary later time point. Units of AP activity were calculated as follows:

$$\text{AP activity} = 1000 \times (OD_{420t} - OD_{420i})/(t \times OD_{600}),$$

where t = reaction time (in minutes, from the addition of Sigma 104 to the addition of K_2HPO_4), $OD_{420t} = OD_{420}$ measurement after a reaction t minutes in length, and OD_{420i} = initial OD_{420} measurement (i.e., prior to the addition of Sigma 104).

Supplemental Data

Supplemental Data include detailed protocols, chemical syntheses, and functional data for the validation of the ABA analogs and can be found at http://www.chembiol.com/cgi/content/full/13/10/1051/DC1/.

Acknowledgments

We thank Dr. W. Wu for help with synthesizing ABA analogs; Shuqiong Lin for assistance in the preparation of azides; S. Bélanger and Y. Rouleau for assistance with screening, western blotting, and immunofluorescence experiments; M. Daroszewska for assistance with analytical chemistry; L. Tessier for assistance in proteomics experiments; R. Bartenschlager (University of Heidelberg, Germany) and L. Supekova (Scripps) for providing template DNA of HCV replicons and Huh-7 cells used in this study; D. Moradpour (University of Freiburg, Germany) for providing a monoclonal antibody against NS5B; and Mahmud Bani (Institute for Biological Sciences, NRC Canada) for assistance with microscopy experiments.

Received: April 24, 2006
Revised: August 8, 2006
Accepted: August 25, 2006
Published: October 20, 2006

References

1. Alter, H.J., and Seeff, L.B. (2000). Recovery, persistence, and sequelae in hepatitis C virus infection: a perspective on long-term outcome. Semin. Liver Dis. *20*, 17–35.
2. C.H. Hagedorn and C.M. Rice, eds. (1999). The Hepatitis C Viruses (Berlin: Springer-Verlag).
3. McCaffrey, A.P., Hashi, K., Meuse, L., Shen, S.L., Lancaster, A.M., Lukavsky, P.J., Sarnow, P., and Kay, M.A. (2002). Determinants of hepatitis C translational initiation in vitro, in cultured cells and mice. Mol. Ther. *5*, 676–684.
4. Lanford, R.E., and Bigger, C. (2002). Advances in model systems for hepatitis C virus research. Virology *293*, 1–9.
5. Wasley, A., and Alter, M.J. (2000). Epidemiology of hepatitis C: geographic differences and temporal trends. Semin. Liver Dis. *20*, 1–16.
6. Bradley, D.W. (1999). Hepatitis viruses: their role in human cancer. Proc. Assoc. Am. Physicians *111*, 588–593.
7. Christie, J.M.L., Chapel, H., Chapman, R.W., and Rosenberg, W.M.C. (1999). Immune selection and genetic sequence variation in core and envelope regions of hepatitis C virus. Hepatology *30*, 1037–1044.
8. Alter, M.J., Margolis, H.S., Krawczynski, K., Judson, F.N., Mares, A., Alexander, W.J., Hu, P.Y., Miller, J.K., Gerber, M.A., Sampliner, R.E., et al. (1992). The natural-history of community-acquired hepatitis-C in the United States. N. Engl. J. Med. *327*, 1899–1905.
9. Lindenbach, B.D., and Rice, C.M. (2005). Unravelling hepatitis C virus replication from genome to function. Nature *436*, 933–938.
10. Reed, K.E., and Rice, C.M. (2000). Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr. Top. Microbiol. Immunol. *242*, 55–84.
11. Chisari, F.V. (2005). Unscrambling hepatitis C virus-host interactions. Nature *436*, 930–932.
12. Zitzmann, N., Mehta, A.S., Carrouee, S., Butters, T.D., Platt, F.M., McCauley, J., Blumberg, B.S., Dwek, R.A., and Block, T.M. (1999). Imino sugars inhibit the formation and secretion of bovine viral diarrhea virus, a pestivirus model of hepatitis C virus: implications for the development of broad spectrum anti-hepatitis virus agents. Proc. Natl. Acad. Sci. USA *96*, 11878–11882.
13. Bartosch, B., Dubuisson, J., and Cosset, F.L. (2003). Infectious hepatitis C virus pseudo-particles containing functional E1–E2 envelope protein complexes. J. Exp. Med. *197*, 633–642.
14. Bigger, C.B., Brasky, K.M., and Lanford, R.E. (2001). DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. J. Virol. *75*, 7059–7066.
15. Su, A.I., Pezacki, J.P., Wodicka, L., Brideau, A.D., Supekova, L., Thimme, R., Wieland, S., Bukh, J., Purcell, R.H., Schultz, P.G., et al. (2002). Genomic analysis of the host response to hepatitis C virus infection. Proc. Natl. Acad. Sci. USA *99*, 15669–15674.
16. Yan, W., Lee, H., Yi, E.C., Reiss, D., Shannon, P., Kwieciszewski, B.K., Coito, C., Li, X.J., Keller, A., Eng, J., et al. (2004). System-based proteomic analysis of the interferon response in human liver cells. Genome Biol. *5*, R54.
17. Dimitrova, M., Imbert, I., Kieny, M.P., and Schuster, C. (2003). Protein-protein interactions between hepatitis C virus nonstructural proteins. J. Virol. *77*, 5401–5414.
18. Garry, R.F., and Dash, S. (2003). Proteomics computational analyses suggest that hepatitis C virus E1 and pestivirus E2 envelope glycoproteins are truncated class II fusion proteins. Virology *307*, 255–265.
19. Majumder, M., Ghosh, A.K., Steele, R., Ray, R., and Ray, R.B. (2001). Hepatitis C virus NS5A physically associates with p53 and regulates p21/waf1 gene expression in a p53-dependent manner. J. Virol. *75*, 1401–1407.
20. Flajolet, M., Rotondo, G., Daviet, L., Bergametti, F., Inchauspe, G., Tiollais, P., Transy, C., and Legrain, P. (2000). A genomic approach of the hepatitis C virus generates a protein interaction map. Gene *242*, 369–379.
21. Lamarre, D., Anderson, P.C., Bailey, M., Beaulieu, P., Bolger, G., Bonneau, P., Bos, M., Cameron, D.R., Cartier, M., Cordingley, M.G., et al. (2003). An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. Nature *426*, 186–189.
22. Soderholm, J., Uehara-Bingen, M., Weis, K., and Heald, R. (2006). Challenges facing the biologist doing chemical genetics. Nat. Chem. Biol. *2*, 55–58.
23. Bredel, M., and Jacoby, E. (2004). Chemogenomics: an emerging strategy for rapid target and drug discovery. Nat. Rev. Genet. *5*, 262–275.
24. Mayer, T.U., Kapoor, T.M., Haggarty, S.J., King, R.W., Schreiber, S.L., and Mitchison, T.J. (1999). Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. Science *286*, 971–974.
25. Gray, N.S., Wodicka, L., Thunnissen, A., Norman, T.C., Kwon, S.J., Espinoza, F.H., Morgan, D.O., Barnes, G., LeClerc, S., Meijer, L., et al. (1998). Exploiting chemical libraries, structure, and genomics in the search for kinase inhibitors. Science *281*, 533–538.
26. Rosania, G.R., Chang, Y.T., Perez, O., Sutherlin, D., Dong, H.L., Lockhart, D.J., and Schultz, P.G. (2000). Myoseverin, a microtubule-binding molecule with novel cellular effects. Nat. Biotechnol. *18*, 304–308.
27. Saghatelian, A., and Cravatt, B.F. (2005). Assignment of protein function in the postgenomic era. Nat. Chem. Biol. *1*, 130–142.
28. Razem, F.A., El-Kereamy, A., Abrams, S.R., and Hill, R.D. (2006). The RNA-binding protein FCA is an abscisic acid receptor. Nature *439*, 290–294.
29. Cutler, A.J., Rose, P.A., Squires, T.M., Loewen, M.K., Shaw, A.C., Quail, J.W., Krochko, J.E., and Abrams, S.R. (2000). Inhibitors of abscisic acid 8′-hydroxylase. Biochemistry *39*, 13614–13624.
30. Abrams, S.R., and Milborrow, B.V. (1991). Synthesis and biological-activity of allenic analogs of abscisic-acid. Phytochemistry *30*, 3189–3195.
31. Abrams, S.R., Rose, P.A., Cutler, A.J., Balsevich, J.J., Lei, B., and Walker-Simmons, M.K. (1997). 8′-Methylene abscisic acid. An effective and persistent analog of abscisic acid. Plant Physiol. *114*, 89–97.
32. Nyangulu, J.M., Galka, M.M., Jadhav, A., Gai, Y., Graham, C.M., Nelson, K.M., Cutler, A.J., Taylor, D.C., Banowetz, G.M., and Abrams, S.R. (2005). An affinity probe for isolation of abscisic acid-binding proteins. J. Am. Chem. Soc. *127*, 1662–1664.

33. Zaharia, L.I., Walker-Simmon, M.K., Rodriguez, C.N., and Abrams, S.R. (2005). Chemistry of abscisic acid, abscisic acid catabolites and analogs. J. Plant Growth Reg. *24*, 274–284.
34. Berger, J., and Moller, D.E. (2002). The mechanisms of action of PPARs. Annu. Rev. Med. *53*, 409–435.
35. Elstner, E., Muller, C., Koshizuka, K., Williamson, E.A., Park, D., Asou, H., Shintaku, P., Said, J.W., Heber, D., and Koeffler, H.P. (1998). Ligands for peroxisome proliferator-activated receptor gamma and retinoic acid receptor inhibit growth and induce apoptosis of human breast cancer cells in vitro and in BNX mice. Proc. Natl. Acad. Sci. USA *95*, 8806–8811.
36. Rakic, B., Sagan, S.M., Noestheden, M., Belanger, S., Nan, X., Evans, C.L., Xie, X.S., and Pezacki, J.P. (2006). Peroxisome proliferator-activated receptor alpha antagonism inhibits hepatitis C virus replication. Chem. Biol. *13*, 23–30.
37. Sagan, S.M., Rouleau, R., Leggiadro, C., Supekova, L., Schultz, P.G., Su, A.I., and Pezacki, J.P. (2006). The influence of cholesterol and lipid metabolism on host cell structure and hepatitis C virus replication. Biochem. Cell Biol. *84*, 67–79.
38. Ye, J., Wang, C.F., Sumpter, R., Brown, M.S., Goldstein, J.L., and Gale, M. (2003). Disruption of hepatitis C virus RNA replication through inhibition of host protein geranylgeranylation. Proc. Natl. Acad. Sci. USA *100*, 15865–15870.
39. Kalgutkar, A.S., Gardner, I., Obach, R.S., Shaffer, C.L., Callegari, E., Henne, K.R., Mutlib, A.E., Dalvie, D.K., Lee, J.S., Nakai, Y., et al. (2005). A comprehensive listing of bioactivation pathways of organic functional groups. Curr. Drug Metab. *6*, 161–225.
40. Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A., and Evans, R.M. (1992). Convergence of 9-cis retinoic acid and peroxisome proliferator signaling pathways through heterodimer formation of their receptors. Nature *358*, 771–774.
41. Hamamoto, S., Fukuda, R., Ishimura, N., Azharul, M., Rumi, K., Kazumori, H., Uchida, Y., Kadowaki, Y., Ishihara, S., and Kinoshita, Y. (2003). 9-cis retinoic acid enhances the antiviral effect of interferon on hepatitis C virus replication through increased expression of type I interferon receptor. J. Lab. Clin. Med. *141*, 58–66.
42. Wang, Q., Chan, T.R., Hilgraf, R., Fokin, V.V., Sharpless, K.B., and Finn, M.G. (2003). Bioconjugation by copper(I)-catalyzed azide-alkyne [3+2] cycloaddition. J. Am. Chem. Soc. *125*, 3192–3193.
43. Bogyo, M., McMaster, J.S., Gaczynska, M., Tortorella, D., Goldberg, A.L., and Ploegh, H. (1997). Covalent modification of the active site threonine of proteasomal β subunits and the *Escherichia coli* homolog HslV by a new class of inhibitors. Proc. Natl. Acad. Sci. USA *94*, 6629–6634.
44. Dick, L.R., Cruikshank, A.A., Grenier, L., Melandri, F.D., Nunes, S.L., and Stein, R.L. (1996). Mechanistic studies on the inactivation of the proteasome by lactacystin: a central role for clastolactacystin β-lactone. J. Biol. Chem. *271*, 7273–7276.
45. Fenteany, G., Standaert, R.F., Lane, W.S., Choi, S., Corey, E.J., and Schreiber, S.L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin. Science *268*, 726–731.
46. Koen, Y.M., and Hanzlik, R.P. (2002). Identification of seven proteins in the endoplasmic reticulum as targets for reactive metabolites of bromobenzene. Chem. Res. Toxicol. *15*, 699–706.
47. Monks, T.J., and Lau, S.S. (1988). Reactive intermediates and their toxicological significance. Toxicology *52*, 1–53.
48. Bardwell, J.C.A., McGovern, K., and Beckwith, J. (1991). Identification of a protein required for disulfide bond formation *in vivo*. Cell *67*, 581–589.
49. Ostermeier, M., De Sutter, K., and Georgiou, G. (1996). Eukaryotic protein disulfide isomerase complements *Escherichia coli dsbA* mutants and increases the yield of a heterologous secreted protein with disulfide bonds. J. Biol. Chem. *271*, 10616–10622.
50. Brickman, E., and Beckwith, J. (1975). Analysis of the regulation of *Escherichia coli* alkaline phosphatase using deletions and ϕ80 transducing phages. J. Mol. Biol. *96*, 307–316.
51. Torriani, A. (1966). Alkaline phosphatase from *Escherichia coli*. In Procedures in Nucleic Acid Research, G.L. Cantoni and R. Davies, eds. (New York: Harper and Row), pp. 224–234.
52. Carvalho, A.P., Fernandes, P.A., and Ramos, M.J. (2006). Similarities and differences in the thioredoxin superfamily. Prog. Biophys. Mol. Biol. *91*, 229–248.
53. Nakamoto, H., and Bardwell, J.C.A. (2004). Catalysis of disulfide bond formation and isomerization in the *Escherichia coli* periplasm. Biochim. Biophys. Acta *1694*, 111–119.
54. Stafford, S.J., and Lund, P.A. (2000). Mutagenic studies on human protein disulfide isomerase by complementation of *Escherichia coli dsbA* and *dsbC* mutants. FEBS Lett. *466*, 317–322.
55. Barbouche, R., Miquelis, R., Jones, I.M., and Fenouillet, E. (2003). Protein-disulfide isomerase-mediated reduction of two disulfide bonds of HIV envelope glycoprotein 120 occurs post-CXCR4 binding and is required for fusion. J. Biol. Chem. *278*, 3131–3136.
56. Barbouche, R., Lortat-Jacob, H., Jones, I.M., and Fenouillet, E. (2005). Glycosaminoglycans and protein disulfide isomerase-mediated reduction of HIV Env. Mol. Pharmacol. *67*, 1111–1118.
57. Fenouillet, E., Barbouche, R., Courageot, J., and Miquelis, R. (2001). The catalytic activity of protein disulfide isomerase is involved in human immunodeficiency virus envelope-mediated membrane fusion after CD4 cell binding. J. Infect. Dis. *183*, 744–752.
58. Gallina, A., Hanley, T.M., Mandel, R., Trahey, M., Broder, C.C., Viglianti, G.A., and Ryser, H.J.P. (2002). Inhibitors of protein-disulfide isomerase prevent cleavage of disulfide bonds in receptor-bound glycoprotein 120 and prevent HIV-1 entry. J. Biol. Chem. *277*, 50579–50588.
59. Gething, M.J., and Sambrook, J. (1992). Protein folding in the cell. Nature *355*, 33–45.
60. Mandel, R., Ryser, H.J.P., Ghani, F., Wu, M., and Peak, D. (1993). Inhibition of a reductive function of the plasma-membrane by bacitracin and antibodies against protein disulfide-isomerase. Proc. Natl. Acad. Sci. USA *90*, 4112–4116.
61. Ryser, H.J.P., Levy, E.M., Mandel, R., and Disciullo, G.J. (1994). Inhibition of Human-Immunodeficiency-Virus infection by agents that interfere with thiol-disulfide interchange upon virus-receptor interaction. Proc. Natl. Acad. Sci. USA *91*, 4559–4563.
62. Tardif, K.D., Mori, K., Kaufman, R.J., and Siddiqui, A. (2004). Hepatitis C virus suppresses the IRE1–XBP1 pathway of the unfolded protein response. J. Biol. Chem. *279*, 17158–17164.
63. Callens, N., Ciczora, Y., Bartosch, B., Vu-Dac, N., Cosset, F.L., Pawlotsky, J.M., Penin, F., and Dubuisson, J. (2005). Basic residues in hyervariable region 1 of hepatitis C virus envelope glycoprotein E2 contribute to virus entry. J. Virol. *79*, 15331–15341.
64. Tellinghuisen, T.L., Marcotrigiano, J., and Rice, C.M. (2005). Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature *435*, 374–379.
65. Penin, F., Dubuisson, J., Rey, F.A., Moradpour, D., and Pawlotsky, J.M. (2004). Structural biology of hepatitis C virus. Hepatology *39*, 5–19.