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Zinc Mesoporphyrin Induces Rapid Proteasomal Degradation of Hepatitis C Nonstructural 5A Protein in Human Hepatoma Cells

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Abstract

Background & Aims—The nonstructural 5A (NS5A) protein of hepatitis C virus (HCV), plays a critical role in HCV replication and is an attractive target for the therapy of HCV infection. So far, little is known about the post-translational regulation of NS5A protein and its precise role in HCV RNA replication. Our objectives were to elucidate the down-regulation of NS5A protein and HCV RNA replication by zinc mesoporphyrin (ZnMP), and the mechanism by which this process occurs.

Methods—Human hepatoma cells expressing HCV proteins were used to investigate the post-translational regulation of ZnMP on NS5A protein by Western blots (WB) and immunoprecipitation (IP). Quantitative RT-PCR (qRT-PCR) was used to determine the effects of ZnMP on HCV RNA replication.

Results—ZnMP selectively and markedly down-regulated NS5A protein levels by increasing degradation of NS5A protein [half life fell from 18.7 h to 2.7 h]. The proteasome inhibitors, epoxomicin and MG132, significantly abrogated degradation of NS5A protein by ZnMP without affecting levels of NS5A in the absence of ZnMP. Analysis of immunoprecipitates with an anti-ubiquitin antibody revealed polyubiquitination of NS5A, suggesting that ZnMP induces ubiquitination of NS5A protein. In addition, 10 μM of ZnMP reduced HCV replication by ~63% in the Con1 replicon cells, ~70% in J6/JFH1 HCV transfected cells, and ~90% in J6/JFH1 HCV infected cells without affecting cell viability.

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Conclusions—ZnMP produces a rapid and profound down-regulation of the NS5A protein by enhancing its polyubiquitination and proteasome-dependent catabolism. Zinc mesoporphyrin may hold promise as a novel agent to treat HCV infection.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause of acute hepatitis and chronic liver disease, including cirrhosis and liver cancer[1]. Current treatments for HCV infection using pegylated interferon (Peg-IFN) and ribavirin (RBV) fail to produce sustained virological responses in fewer than 50% of adequately treated patients infected with HCV genotype 1, and have numerous unpleasant side effects. These side-effects and the rather high costs, and required duration of such treatment limit its use. Thus, it is too costly for most patients in developing countries and many in developed countries to afford. Clearly, more effective strategies to treat HCV infection are urgently needed.

The hepatitis C virion contains a 9.6-kb positive single-strand RNA genome which encodes a single polyprotein of approximately 3010 amino acids, that is then processed into structural (C, E1, E2) and nonstructural (NS2, NS3, NS4A, NS4B, NS5A and NS5B) proteins2¯5. The nonstructural 5A (NS5A) protein, a major component of HCV proteins, is a 447-amino-acid phosphorylated zinc-metalloprotein with largely unknown functions6, 7. Recent evidence has shown that NS5A plays a critical role in the replication of HCV, both directly, with regard to viral RNA replication, and indirectly, by modulating the host cell environment to favor the virus6¯8. NS5A has been shown to interact with nonstructural 5B (NS5B) protein, the viral RNA-dependent RNA polymerase, and this interaction is essential for maintenance of subgenomic replications in Huh-7 replicon cells9, 10. NS5A binds to the 3′-ends of HCV positive strand RNA with high affinity, and this binding is essential for genome replication and infection11. A recent study revealed that a cellular microRNA-196 (miR-196) directly targets NS5A and is able to substantially attenuate viral replication in JFH1 replicon Huh-7 cells12. The most recent study from Hughes et al reported that domain III of NS5A is implicated in both RNA replication and assembly of hepatitis C virus particles in JFH1-infected cells13. Although precise functions of NS5A in both HCV genomic RNA replication and modulation of physiology of the host cell remain unclear, targeting NS5A is an attractive emerging strategy for the therapy of HCV infection8, 14.

Zinc mesoporphyrin (ZnMP) is a non-heme metalloporphyrin and a synthetic heme analogue with a central zinc of the mesoporphyin macrocycle. ZnMP has been demonstrated as a potent inhibitor of heme oxygenase (HO) and proposed as a therapeutic agent for severe unconjugated hyperbilirubinemia (e.g., Crigler-Najjar syndrome type II) and for prolonging effects of heme in therapy of acute porphyric syndromes15, 16. Recent studies from our and other laboratories have shown that ZnMP17, tin mesoporphyrin (SnMP)18 and heme19 induce ubiquitination and proteasomal degradation of Bach1, a transcriptional repressor of the heme oxygenase 1 (HO-1) gene, in human hepatoma cells and NIH3T3 cells as one of the mechanisms by which the HO-1 gene is up-regulated. In addition, we have recently reported that cobalt protoporphyrin (CoPP) down-regulates HCV core protein and suppresses HCV replication in human hepatoma cells stably expressing HCV proteins20. So far, little is known about the ubiquitin-proteasome degradation of NS5A protein and its role in HCV RNA replication. In this study, we show down-regulation of NS5A protein by ZnMP and the molecular mechanism involved in this process. We demonstrate that ZnMP decreases the level of NS5A protein at the post-translational level by modulating protein degradation through a proteasome-ubiquitin degradation pathway. In addition, ZnMP suppress HCV RNA replication. We propose that zinc porphyrin-mediated ubiquitin-proteasome degradation pathway may play a role in affecting the HCV RNA replication

through controlling the amount of NS5A protein. The observations presented herein suggest that zinc porphyrins may be of value in treatment of HCV infection.

MATERIALS AND METHODS

See Supplemental Materials and Methods for more details.

Cell Lines

The 9–13, CNS3 and Huh-7/T7 cell lines were kindly provided by R. Bartenschlager (University of Heidelberg, Heidelberg, Germany). 9–13 subgenomic replicon cells, containing a replicating HCV nonstructural region of the Con1 isolate, stably express HCV NS3 to NS5B21. CNS3 cells stably express HCV core to NS3. Huh-7/T7 cells constitutively express the bacteriophage T7 RNA polymerase22. Cell line Huh-7.5, a highly permissive, alpha interferon-cured Huh-7 derivative was kindly provided by C. M. Rice (The Rockefeller University, New York, NY). The Con1 genotype 1b HCV replicon cell line was from Apath LLC (St, Louis, MO). The Con1 cell line is a Huh-7.5 cell population containing the full-length HCV genotype 1b replicon 23, 24.

Constructs and Transfection

The pFK-Con1/GDD and pFK-Con1/GND constructs (genotype 1b) were kind gifts of Dr. R. Bartenschlager (University of Heidelberg, Heidelberg, Germany). The pFK-Con1/GDD construct containing the wild type Con1 genome was described[21] and pFK-Con1/GND construct was a replication-deficient variant of pFK-Con1/GDD with a single amino substitution, which changed the GDD motif of the NS5B polymerase active site to GND25. Transfection of pFK-Con1/GDD or pFK-Con1/GND was performed as described in Suppl. Materials and Methods.

In Vitro Transcription, HCV RNA Transfection and Infection

The HCV infectious clone pJ6/JFH1 was generously provided by Dr. C. Rice (the Rockefellller University, New York, NY). The full-length chimeric genome was constructed with the use of the core-NS2 gene regions from the infectious J6 (genotype 2a) and NS3-NS5B gene regions from the infectious JFH1 (genotype 2a) as described by Lindenbach et al[23]. To generate HCV J6/JFH1 RNA, the pJ6/JFH1 plasmid was linearized with *Xba*I, and purified by ethanol precipitation, digestion with proteinase K, and phenol-chloroform extraction. The linearized plasmid was used as a template for *in vitro* transcription using the MEGAscript T7 kit (Ambion, Austin, TX). For HCV RNA transfection, Huh-7.5 cells were plated in 24-well plates one day prior to transfection and transfected at 70~80% confluence. Cells were transfected at an RNA/lipofectamine ratio of 1:2 by using 2 μg/well of HCV RNA and 4 uL/well Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 48 h. For HCV infection, cell culture supernatants from the cells transfected with HCV RNA for 48 h were collected and filtered through a 0.20 μm filter, and infected naïve Huh-7.5 cells in 24-well plates for 72 h. Total RNA and protein were extracted for subsequent RT-PCR and Western blot measurements.

Western Blots, Quantitative RT-PCR and Immunoprecipitation (IP)

Western blot analysis and quantitative RT-PCR were performed using the standard protocols of our laboratory as described previously[17]. Immunoprecipitation was carried out according to the manufacturer's protocol from Santa Cruz (Santa Cruz, CA). Antibodies used are described in Supplemental Material and Methods.

Protein half-life determination

9–13 cells were incubated with 100 ug/mL cycloheximide (CHX) in the presence or absence of 10 uM ZnMP. Western blots were performed using anti-HCV NS5A and anti-GAPDH antibodies. Band intensities of Western blots were measured by densitometric analysis. GAPDH bands were used as internal controls to correct for protein loadings.

RESULTS

Down-regulation of NS5A protein levels by ZnMP

Earlier studies from our and other laboratories have shown that heme or cobalt protoporphyrin (CoPP) significantly reduced HCV20 or HBV26 core proteins. However, little attention has been given to the effect of ZnMP on the HCV proteins. To investigate whether ZnMP down-regulates the HCV proteins, we measured NS5A protein levels in 9–13 and Con1 cells, and core protein levels in CNS3 cells exposed to different concentrations of ZnMP (0, 1, 5, 10 μM) for 4 h, ZnMP led to a marked decrease of NS5A protein levels in 9–13 and Con1 cells in a dose-dependent fashion (Fig. 1A & B).

The effect was selective and specific: there were no detectable effects of ZnMP on HCV core protein levels in CNS3 cells and in Huh-7/T7 cells transfected with pFK-Con1/GND, a plasmid encoding a replication deficient variant of Con1 (Fig. 1C & D). We also observed that protein levels were not affected by 10 μM free mesoporphyrin or $ZnCl_2$ (Fig. 2A). Tin mesoporphyrin (SnMP), another competitive HO inhibitor, has been reported recently to down-regulate Bach1 protein levels and induce the HO-1 gene expression in NIH 3T3 cells. We thus performed comparative studies on ZnMP vs SnMP down-regulation of NS5A in 9–13 cells. As shown in Fig. 2B, ZnMP markedly and rapidly decreased NS5A protein levels after exposure to ZnMP for as little as 2 h. In contrast, no detectable effects of SnMP on NS5A protein levels were observed. In addition, the potent zinc chelator, N, N, N, N-tetrakis-(2-pyridylmethyl) ethylenediamine (TEPN) did not affect ZnMP-mediated profound down-regulation of NS5A protein levels in 9–13 cells (Fig. 3).

ZnMP decreases the stability of NS5A protein

Next we examined whether down-regulation of NS5A protein by ZnMP occurs at a post-translational level. As shown in Fig. 4, NS5A protein levels in 9–13 cells treated with ZnMP and cycloheximide (CHX) were greatly and rapidly reduced. NS5A protein levels in 9–13 cells that were not treated with ZnMP were also decreased by CHX, but to a much lesser extent. Specifically, ZnMP at a concentration of 10 μM decreased the NS5A protein half life ($t_{1/2}$) from 18.7 h to 2.7 h (Fig. 4B & C).

Proteasome inhibitors abrogate the degradation of NS5A by ZnMP

To understand whether degradation of NS5A protein by ZnMP is proteasome dependent, 9–13 cells were treated with ZnMP (5, 10 μM) and selected proteasome inhibitors, epoxomicin (5, 10 μM) and MG132 (10, 20 μM). We found that epoxomicin (5, 10 μM) and MG132 (10, 20 μM) completely abrogated the degradation of NS5A in cells exposed to a lower concentration of ZnMP (5 μM), and cells treated with ZnMP (10 μM) and epoxomicin (5, 10 μM) or MG132 (10, 20 μM) displayed significant, although less than complete, diminution of the degradation of NS5A by ZnMP (Fig. 5A & B), however, epoxomicin or MG132 did not affect NS5A protein levels in the absence of ZnMP in 9–13 cells (Fig. 5C), suggesting that the proteasome-dependent degradation pathway is involved in ZnMP-mediated NS5A breakdown. The highest concentration of epoxomicin used in these experiments was 10 μM, because cells exposed to 20 μM epoxomicin failed to grow well, indicating that this concentration of epoxomicin was toxic to the cells.

ZnMP induces polyubiquitination of NS5A protein

To gain insight into the mechanism by which ZnMP mediates degradation of NS5A protein, we examined whether ZnMP induce polyubiquitination of NS5A [(Ub)n-NS5A]. After treating cells with ZnMP, or with vehicle (DMSO) alone as control, cell lysates were prepared and were subjected to immunoprecipitation (IP) of NS5A with anti-NS5A monoclonal antibody. An immunoblotting (IB) analysis of the precipitates with an anti-ubiquitin antibody revealed polyubiquitination of NS5A [(Ub)n-NS5A] following ZnMP treatment (Fig. 6B). In contrast, it did not show a significant level of ubiquitination under control condition (Fig. 6B). Immunoblot analysis with anti-NS5A antibody confirmed that NS5A proteins were immunoprecipitated (Fig. 6C) and that higher-molecular-weight bands may represent ubiquitylated forms of the NS5A protein (Fig. 6C). The results suggest that ZnMP induce polyubiquitination of NS5A which contributes to the degradation of NS5A by ZnMP.

ZnMP displays anti-viral activity

To evaluate whether ZnMP-mediated degradation of NS5A may play a role in inhibiting HCV replication, Con1 full-length HCV replicon cells were treated with different concentrations of ZnMP for 24 h. As expected, vehicle (DMSO) alone did not alter the amounts of HCV replicon RNA, whereas treatment with ZnMP resulted in a dose-dependent reduction in viral RNA (Fig. 7A), and protein levels (Fig. 7B), suggesting that ZnMP-mediated rapid degradation of NS5A may lead to reduction of HCV RNA replication, and subsequent decrease in HCV protein expression. And then we asked if NS5A is an actual target of ZnMP and the effects of ZnMP on HCV RNA replication and core protein levels are secondary to ZnMP-mediated rapid degradation of NS5A. To this end, we performed parallel experiments with HCV proteins expressed from a DNA plasmid pFK-Con1/GND in Huh-7/T7 cells, where their expression would not be linked to viral RNA polymerase but only to T7 RNA polymerase. ZnMP markedly decreased NS5A protein levels in a dose-dependent fashion, whereas HCV core protein levels remained unaffected after 24 h of ZnMP treatment (Fig. 7C). We further observed that ZnMP resulted in reduction of core in the system that HCV proteins were expressed from pFK-Con1/GDD in Huh-7/T7 cells, where their expression would be partly linked to viral RNA polymerase (Fig. 7D), however, the reduction of core was much less than the effect in Con1 replicon system, where expression of HCV proteins were linked to viral RNA polymerase.

Next, we used the JFH1-based HCV cell culture system to investigate whether ZnMP down-regulates NS5A protein and displays anti-viral activity in this novel JFH1-based (genotype 2a) HCVcc system. We measured NS5A and core protein levels in J6/JFH1 transfected Huh-7.5 cells treated with indicated concentrations of ZnMP for 4 or 24 h. ZnMP led to a rapid and profound decrease of NS5A protein levels, while core protein levels were not affected after 4 h of ZnMP treatment and showed a decrease after 24 h exposure to ZnMP (Fig. 8A-D). To further examine whether ZnMP inhibits HCV RNA replication/infection in J6/JFH1 transfected and infected cell culture system, we analyzed HCV RNA expression after ZnMP treatment. 10 μM of ZnMP markedly decreased HCV RNA levels by ~70% in HCV-transfected cells and ~90% in HCV-infected cells (Fig. 8E & F).

To exclude the possibility that the reduction in HCV viral replication could be a non-specific effect of ZnMP to cause cytotoxicity of treated cells, cells were exposed to ZnMP (0, 1, 5, 10 μM) for 2, 6, or 24 h. As shown in Suppl. Fig. 2, ZnMP treatments did not significantly affect cell viability, suggesting that the effects of ZnMP on HCV viral RNA and protein expression were not due to cytotoxic effects.

DISCUSSION

The major findings of this paper are as follows: 1) ZnMP, but not the free mesoporphyrin nor $ZnCI_2$ down-regulates NS5A protein levels in a dose–dependent fashion in human hepatoma cells stably expressing HCV proteins (Fig. 1, 2 & 3); 2) ZnMP reduces the stability of NS5A protein by decreasing its half life from 18.7 h to 2.7 h, suggesting that the effect of ZnMP on NS5A occurs at a post-translational level (Fig. 4); 3) proteasome inhibitors, epoxomicin and MG132, block the degradation of NS5A (Fig. 5), indicating that down-regulation of NS5A by ZnMP is proteasome dependent; 4) ZnMP significantly induces polyubiquitination of NS5A, supporting the hypothesis that the proteasome-ubiquitin degradation pathway is involved in down-regulation of NS5A (Fig. 6); 5) ZnMP significantly reduces HCV RNA replication in Con1 replicon cells, JFH1-based HCV transfected cells, and JFH1-based HCV infected cells, suggesting that ZnMP-mediated degradation of NS5A may play a part in inhibiting HCV replication/infection (Fig. 7 & 8). 6) There is no evidence that ZnMP affects HCV core protein levels in CNS3 cells (Fig. 1C), in Huh-7/T7 cells transfected with pFK-Con1/GND (Fig. 1D & 7C), a plasmid encoding a replication deficient variant of Con1 (Fig. 1D), indicating that the effect of ZnMP on NS5A is selective and specific.

In this study, three different cell lines that stably express HCV proteins and JFH1-based HCV cell culture system were used (Suppl. Fig. 1). ZnMP decreased NS5A protein levels in Con1-based replicon cells and JFH1-based HCV cell culture system but did not show significant down-regulation of core protein in CNS3 (Fig. 1) and Huh-7/T7 cells transfected with pFK-Con1/GND (Fig.1D & 7C). ZnMP at concentrations of 10 μM was not toxic (Suppl. Fig. 2) and the maximal concentration of ZnMP used in this study was at 10 μM. Therefore it is clear that the down-regulation of ZnMP on NS5A and HCV RNA repliction are not due to a general, non-specific toxic effect, but due to its ability to activate the ubiquitin-proteasomal pathway of NS5A protein catabolism.

In recent years, it has become increasingly clear that that NS5A plays a key role in HCV RNA replication by participating in polyprotein cleavage, interferon response and cellular signaling pathways8, 14. HCV NS5A includes domain I (amino acids 1–213), domain II (amino acids 250–342), and domain III (amino acids 355–447)8, 27. The N-terminal domain (domain I) contains a zinc binding motif, which is a vital component of the HCV replication7. Domain II, containing an interferon sensitivity-determining region (amino acids 237–276), is associated with IFN resistance and lack of response to treatment in genotype1a-infected patients28. In this report, we observed that ZnMP induces markedly down-regulation of NS5A without affecting core protein and significantly reduces HCV RNA replication. We thus propose that the reduction of HCV RNA replication occurs primarily through controlling the amount of HCV NS5A protein by ZnMP. These results also provide strong evidence to support the novel strategy for treatment of HCV infection by targeting NS5A protein levels.

Ubiquitin (Ub) was first identified as a highly-conserved small protein in eukaryotic cells that is composed of 76 amino acids with a predicted molecular weight of 8.5 kDa29, 30. The ubiquitin-proteasome degradation pathway has been well accepted as an important regulatory system in many cellular processes such as cell cycle, DNA repair, embryogenesis, the regulation of transcription and apoptosis29–31. In the ubiquitin-proteasome pathway, protein substrates are first marked for degradation by covalent linkage to multiple molecules of ubiquitin (polyubiquitination) and then are hydrolyzed by the 26 S proteasome, a 2000 kDa ATP-dependent proteolytic complex. We previously reported that ZnMP produces profound degradation of Bach1 protein in a proteasome-dependent manner as one of molecular mechanisms by which ZnMP up-regulates the HO-1 gene expression[17]. Others

have reported that HCV proteins, such as NS5B32, core33, 34, the unglycosylated cytosolic form of E2[35], NS2[36], and F protein[37] are degraded through the ubiquitin-proteasome pathway. To our knowledge, the present study is the first report showing the degradation of NS5A protein and reduction of HCV RNA replication by degradation of NS5A. Taken together, these studies suggest that the ubiquitin-proteasome pathway plays a role in the HCV life cycle or viral pathogenesis. In addition, our data provide a rationale to develop antiviral drugs that increases ubiquitination of NS5A.

ZnMP induces polyubiquitination of NS5A and display anti-viral activity, we found ZnMP an attractive choice, and we chose to use mainly ZnMP because of the profound effects on Bach1[17], because mesoporphyrin derivatives lack reactive vinyl groups and are therefore more chemically stable than protoporphyrin derivatives[38], and because it is taken up by intact liver cells far better than other zinc derivatives such as bisglycol-deuteroporphyrin congener[39]. Zinc porphyrins, under some circumstances, may be toxic to hematopoietic cells in vitro[40]. There was nothing to suggest cytotoxicity to liver cells in this work (Suppl. Fig. 2). In addition, earlier work showed no evidence of toxicity of intravenously administrated ZnMP in intact rats. The ZnMP in these animals partitioned mainly to liver and spleen and inhibited heme oxygenase activity particularly in these organs. In this study, we chose to administer ZnMP bound to human serum albumin (HSA), because like other metalloporphyrins, ZnMP has limited solubility in physiologic buffer, but its solubility and uptake into liver cells are greatly enhanced by albumin binding[41, 42] and because ZnMP, when administered as an albumin complex, is nontoxic and is taken up preferentially by the liver and spleen43.

In conclusion, we have found that ZnMP, but not the free mesoporphyrin nor the metallic ion itself induces post-translational down-regulation of HCV NS5A protein in an ubiquitin-proteasome degradation pathway. Our results indicate that ZnMP significantly suppresses HCV viral replication in the Con1 full length replicon Huh-7.5 cells and JFH1-based HCV cell culture system, suggesting that ZnMP may hold promise as a novel agent to treat HCV infection. We propose that ZnMP-mediated ubiquitin-proteasome degradation pathway may play a role in affecting the HCV RNA replication through controlling the amount of NS5A protein. Our studies also raise many important new questions that need to be answered in future. Further studies are underway in our laboratory, designed to unravel the molecular mechanism that underlies the effect of ZnMP to trigger ubiquitination of Bach1 or HCV NS5A, as well as to search for other zinc derivatives, which might display greater effects on NS5A degradation and HCV RNA replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

BCA	Bicinchoninic acid

CoPP	cobalt protoporphyrin
CHX	Cycloheximide
DMSO	Dimethyl sulfoxide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HO	Heme oxygenase
HCV	Hepatitis C virus
IB	Immunoblotting
IFN-α	Interferon-alpha
IP	Immunoprecipitation
JFH1	Japanese fulminant hepatitis 1
NS5A	Nonstructural protein 5a of HCV
NTR	non-translated region
PVDF	Polyvinylidene fluoride
qRT-PCR	Quantitative real time polymerase chain reaction
SDS	Sodium dodecyl sulfate
SnMP	Tin mesoporphyrin
TEPN	N, N, N, N-tetrakis-(2-pyridylmethyl) ethylenediamine
ZnMP	Zinc mesoporphyrin

References

1. Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 2005;5:558–67. [PubMed: 16122679]
2. Rosenberg S. Recent advances in the molecular biology of hepatitis C virus. J Mol Biol 2001;313:451–64. [PubMed: 11676530]
3. Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science 1989;244:359–62. [PubMed: 2523562]
4. Lohmann V, Koch JO, Bartenschlager R. Processing pathways of the hepatitis C virus proteins. J Hepatol 1996;24:11–9. [PubMed: 8836884]
5. Bartenschlager R, Lohmann V. Replication of hepatitis C virus. J Gen Virol 2000;81:1631–48. [PubMed: 10859368]
6. Tellinghuisen TL, Marcotrigiano J, Gorbalenya AE, Rice CM. The NS5A protein of hepatitis C virus is a zinc metalloprotein. J Biol Chem 2004;279:48576–87. [PubMed: 15339921]
7. Tellinghuisen TL, Marcotrigiano J, Rice CM. Structure of the zinc-binding domain of an essential component of the hepatitis C virus replicase. Nature 2005;435:374–9. [PubMed: 15902263]
8. Szabo G. Hepatitis C virus NS5A protein--a master regulator? Gastroenterology 2006;130:995–9. [PubMed: 16530536]
9. Shirota Y, Luo H, Qin W, Kaneko S, Yamashita T, Kobayashi K, Murakami S. Hepatitis C virus (HCV) NS5A binds RNA-dependent RNA polymerase (RdRP) NS5B and modulates RNA-dependent RNA polymerase activity. J Biol Chem 2002;277:11149–55. [PubMed: 11801599]
10. Shimakami T, Hijikata M, Luo H, Ma YY, Kaneko S, Shimotohno K, Murakami S. Effect of interaction between hepatitis C virus NS5A and NS5B on hepatitis C virus RNA replication with the hepatitis C virus replicon. J Virol 2004;78:2738–48. [PubMed: 14990694]

11. Huang L, Hwang J, Sharma SD, Hargittai MR, Chen Y, Arnold JJ, Raney KD, Cameron CE. Hepatitis C virus nonstructural protein 5A (NS5A) is an RNA-binding protein. J Biol Chem 2005;280:36417–28. [PubMed: 16126720]
12. Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M. Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature 2007;449:919–22. [PubMed: 17943132]
13. Hughes M, Griffin S, Harris M. Domain III of NS5A contributes to both RNA replication and assembly of hepatitis C virus particles. J Gen Virol 2009;90:1329–34. [PubMed: 19264615]
14. Macdonald A, Harris M. Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 2004;85:2485–502. [PubMed: 15302943]
15. Russo SM, Pepe JA, Cable EE, Lambrecht RW, Bonkovsky HL. Repression of ALA synthase by heme and zinc-mesoporphyrin in a chick embryo liver cell culture model of acute porphyria. Eur J Clin Invest 1994;24:406–15. [PubMed: 7957494]
16. Dover SB, Moore MR, Fitzsimmons EJ, Graham A, McColl KE. Tin protoporphyrin prolongs the biochemical remission produced by heme arginate in acute hepatic porphyria. Gastroenterology 1993;105:500–6. [PubMed: 8335204]
17. Hou W, Shan Y, Zheng J, Lambrecht RW, Donohue SE, Bonkovsky HL. Zinc mesoporphyrin induces rapid and marked degradation of the transcription factor Bach1 and up-regulates HO-1. Biochim Biophys Acta 2008;1779:195–203. [PubMed: 18325350]
18. Abate A, Zhao H, Wong RJ, Stevenson DK. The role of Bach1 in the induction of heme oxygenase by tin mesoporphyrin. Biochem Biophys Res Commun 2007;354:757–63. [PubMed: 17257585]
19. Zenke-Kawasaki Y, Dohi Y, Katoh Y, Ikura T, Ikura M, Asahara T, Tokunaga F, Iwai K, Igarashi K. Heme induces ubiquitination and degradation of the transcription factor Bach1. Mol Cell Biol 2007;27:6962–71. [PubMed: 17682061]
20. Shan Y, Zheng J, Lambrecht RW, Bonkovsky HL. Reciprocal effects of micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. Gastroenterology 2007;133:1166–74. [PubMed: 17919492]
21. Lohmann V, Korner F, Koch J, Herian U, Theilmann L, Bartenschlager R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. Science 1999;285:110–3. [PubMed: 10390360]
22. Appel N, Pietschmann T, Bartenschlager R. Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. J Virol 2005;79:3187–94. [PubMed: 15709040]
23. Lindenbach BD, Evans MJ, Syder AJ, Wolk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623–6. [PubMed: 15947137]
24. Blight KJ, McKeating JA, Marcotrigiano J, Rice CM. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. J Virol 2003;77:3181–90. [PubMed: 12584342]
25. Krieger N, Lohmann V, Bartenschlager R. Enhancement of hepatitis C virus RNA replication by cell culture-adaptive mutations. J Virol 2001;75:4614–24. [PubMed: 11312331]
26. Protzer U, Seyfried S, Quasdorff M, Sass G, Svorcova M, Webb D, Bohne F, Hosel M, Schirmacher P, Tiegs G. Antiviral activity and hepatoprotection by heme oxygenase-1 in hepatitis B virus infection. Gastroenterology 2007;133:1156–65. [PubMed: 17919491]
27. Shelton H, Harris M. Hepatitis C virus NS5A protein binds the SH3 domain of the Fyn tyrosine kinase with high affinity: mutagenic analysis of residues within the SH3 domain that contribute to the interaction. Virol J 2008;5:24. [PubMed: 18267011]
28. Reed KE, Xu J, Rice CM. Phosphorylation of the hepatitis C virus NS5A protein in vitro and in vivo: properties of the NS5A-associated kinase. J Virol 1997;71:7187–97. [PubMed: 9311791]
29. Hochstrasser M. Ubiquitin, proteasomes, and the regulation of intracellular protein degradation. Curr Opin Cell Biol 1995;7:215–23. [PubMed: 7612274]
30. Ciechanover A. The ubiquitin-proteasome proteolytic pathway. Cell 1994;79:13–21. [PubMed: 7923371]
31. Finley D, Ciechanover A, Varshavsky A. Ubiquitin as a central cellular regulator. Cell 2004;116:S29–32. 2. p following S32. [PubMed: 15055578]

32. Gao L, Tu H, Shi ST, Lee KJ, Asanaka M, Hwang SB, Lai MM. Interaction with a ubiquitin-like protein enhances the ubiquitination and degradation of hepatitis C virus RNA-dependent RNA polymerase. J Virol 2003;77:4149–59. [PubMed: 12634373]
33. Suzuki R, Tamura K, Li J, Ishii K, Matsuura Y, Miyamura T, Suzuki T. Ubiquitin-mediated degradation of hepatitis C virus core protein is regulated by processing at its carboxyl terminus. Virology 2001;280:301–9. [PubMed: 11162844]
34. Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, Chiba T, Tanaka K, Suzuki R, Suzuki T, Miyamura T, Matsuura Y. Proteasome activator PA28gamma-dependent nuclear retention and degradation of hepatitis C virus core protein. J Virol 2003;77:10237–49. [PubMed: 12970408]
35. Pavio N, Taylor DR, Lai MM. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. J Virol 2002;76:1265–72. [PubMed: 11773402]
36. Franck N, Le Seyec J, Guguen-Guillouzo C, Erdtmann L. Hepatitis C virus NS2 protein is phosphorylated by the protein kinase CK2 and targeted for degradation to the proteasome. J Virol 2005;79:2700–8. [PubMed: 15708989]
37. Xu Z, Choi J, Lu W, Ou JH. Hepatitis C virus f protein is a short-lived protein associated with the endoplasmic reticulum. J Virol 2003;77:1578–83. [PubMed: 12502871]
38. Vreman HJ, Gillman MJ, Stevenson DK. In vitro inhibition of adult rat intestinal heme oxygenase by metalloporphyrins. Pediatr Res 1989;26:362–5. [PubMed: 2797950]
39. Cable EE, Cable JW, Bonkovsky HL. Repression of hepatic delta-aminolevulinate synthase by heme and metalloporphyrins: relationship to inhibition of heme oxygenase. Hepatology 1993;18:119–27. [PubMed: 8325603]
40. Lutton JD, Abraham NG, Drummond GS, Levere RD, Kappas A. Zinc porphyrins: potent inhibitors of hematopoieses in animal and human bone marrow. Proc Natl Acad Sci U S A 1997;94:1432–6. [PubMed: 9037070]
41. Sinclair PR, Bement WJ, Gorman N, Liem HH, Wolkoff AW, Muller-Eberhard U. Effect of serum proteins on haem uptake and metabolism in primary cultures of liver cells. Biochem J 1988;256:159–65. [PubMed: 3223898]
42. Sinclair PR, Bement WJ, Healey JF, Gorman N, Sinclair JF, Bonkovsky HL, Liem HH, Muller-Eberhard U. Effects of hemopexin on heme-mediated repression of 5-aminolevulinate synthase and induction of heme oxygenase in cultured hepatocytes. Hepatology 1994;20:741–6. [PubMed: 8076930]
43. Russo SM, Pepe JA, Donohue S, Cable EE, Lambrecht RW, Bonkovsky HL. Tissue distribution of zinc-mesoporphyrin in rats: relationship to inhibition of heme oxygenase. J Pharmacol Exp Ther 1995;272:766–74. [PubMed: 7853192]

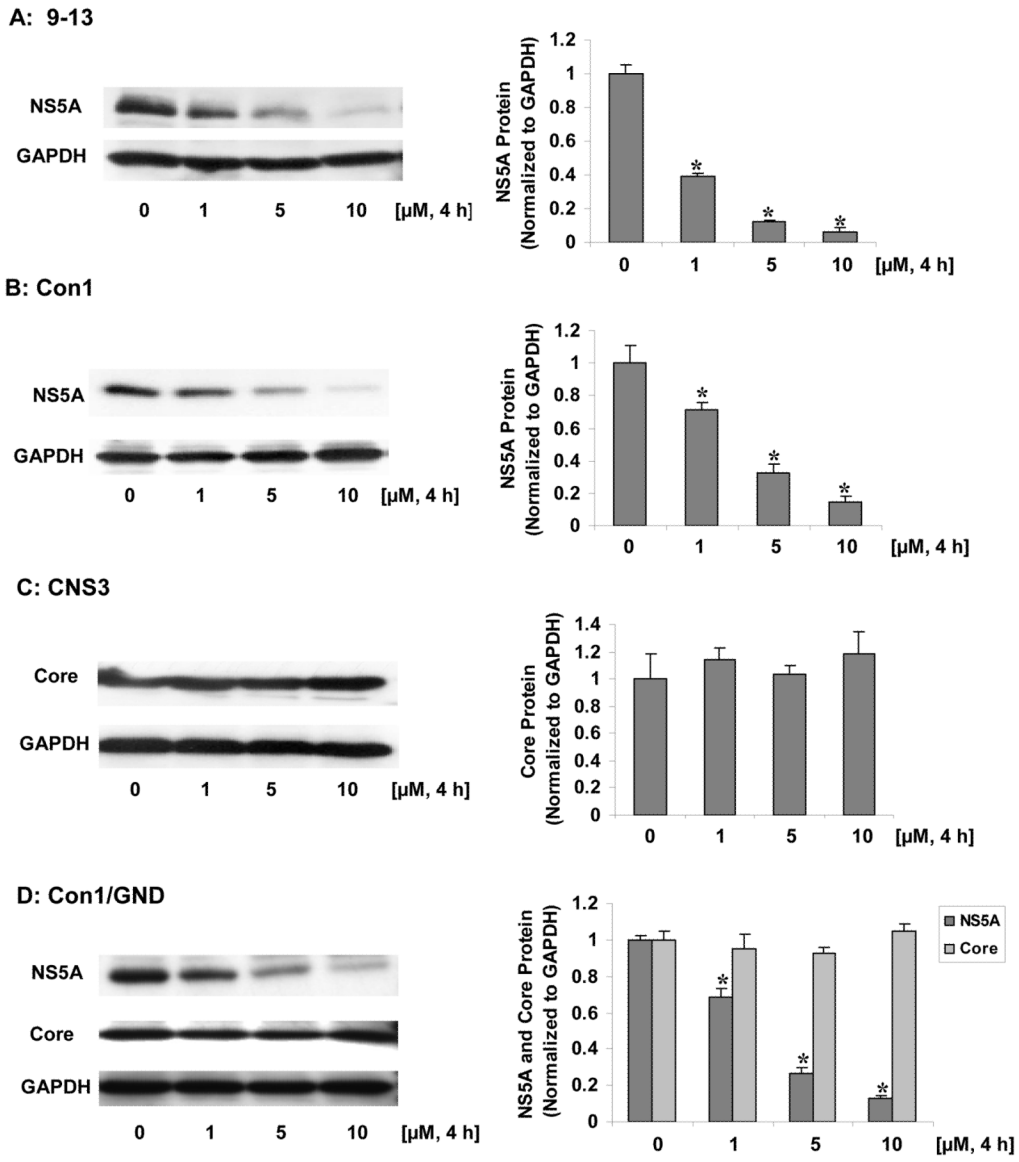


Figure 1. Dose-dependent down-regulation of HCV NS5A but not HCV core protein by ZnMP

Cells were treated with different concentrations of ZnMP (0, 1, 5, 10 μM) for 4 hours, after which cells were harvested and total proteins were isolated for Western blot analysis. The bar graphs show quantitative results. The amounts of NS5A or core protein levels were normalized to GAPDH which did not vary with treatment. Values for cells treated with vehicle only were set equal to 1. Data are presented as means ± SE from triplicate samples. * differs from vehicle only, $P<0.05$. **(A)** ZnMP decreased NS5A protein levels in 9–13 cells. **(B)** ZnMP down-regulated NS5A protein levels in Con1 cells. **(C)** ZnMP did not alter core protein levels in CNS3 cells. **(D)** ZnMP decreased NS5A protein levels, whereas HCV core protein levels remained unchanged in Huh-7/T7 cells transfected with pFK-Con1/GND. Huh-7/T7 cells, stably expressing the T7 RNA polymerase, were transfected with 0.8 ug/well of pFK-Con1/GND by Lipofectamine and Plus Reagent. 48 h after transfection, cells were treated with ZnMP (0, 1, 5, 10 μM) for 4 h. The levels of HCV NS5A, core and GAPDH protein were measured by Western blots.

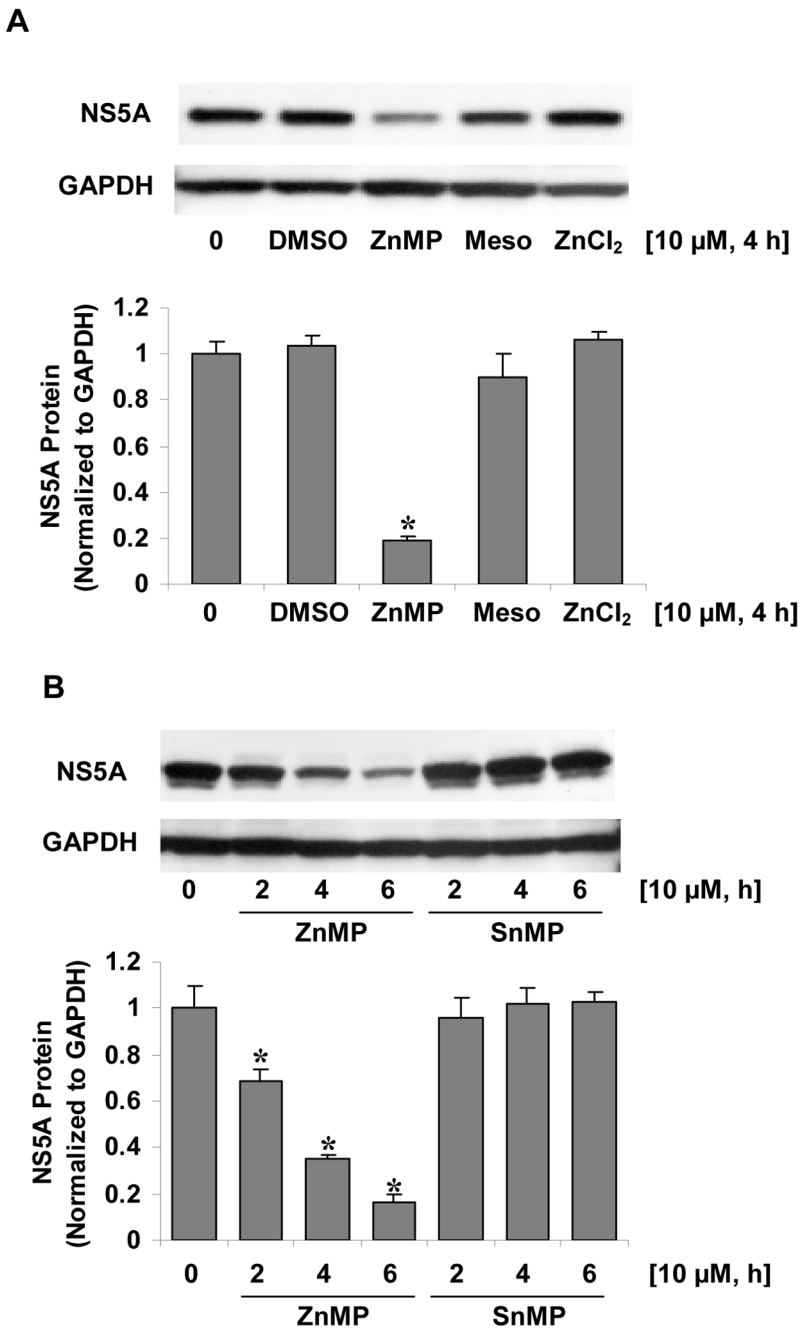


Figure 2. Specificity of ZnMP-mediated down-regulation of NS5A protein levels

(A) 9–13 cells were exposed to ZnMP, free mesoporphyrin (Meso) or $ZnCl_2$ (all at 10 μM) for 4 hours. Western blot was performed using anti-HCV NS5A and GAPDH specific antibodies. **(B)** 9–13 cells were treated with 10 μM ZnMP or with 10 μM SnMP for indicated times (0, 2, 4, 6 h). The levels of HCV NS5A and GAPDH protein were measured by Western blots. The bar graphs show quantitative results. The relative amounts of NS5A protein levels were normalized to those for GAPDH, which did not vary with treatment. Values for cells treated with vehicle only were set equal to 1. Data are presented as means ± SE from triplicate samples. * differs from vehicle only, $P<0.05$.

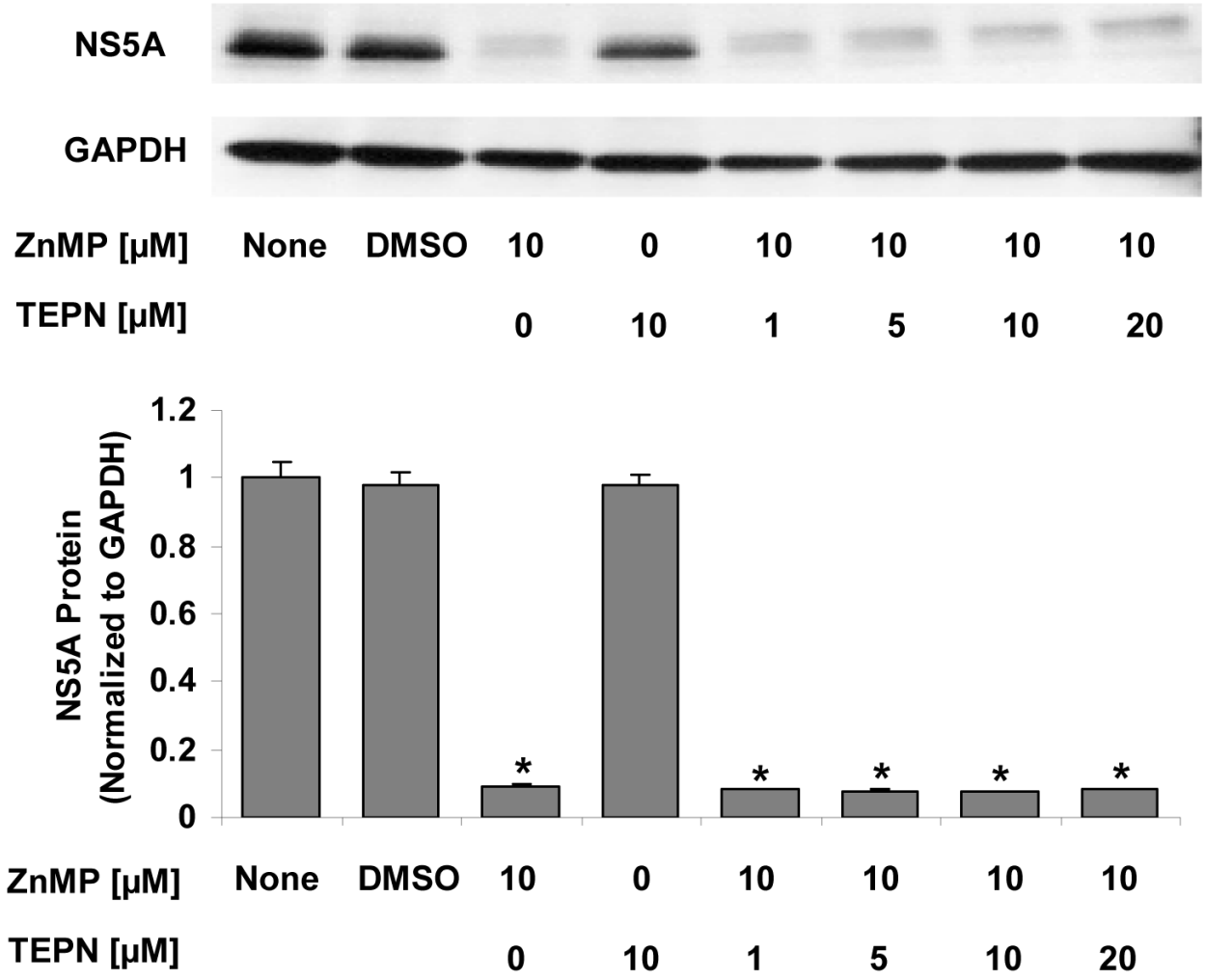


Figure 3. Effect of zinc chelator on NS5A protein levels in 9–13 cells

9–13 cells were treated with indicated concentrations of TEPN 30 min before ZnMP treatment, the cells were subsequently exposed to ZnMP or to vehicle (DMSO) alone as control for 4 h. NS5A and GAPDH protein levels were measured by Western blot. The bar graphs show quantitative results. The relative amounts of NS5A protein were normalized to those for GAPDH, which did not vary with treatment. The band intensity of NS5A from vehicle alone was set equal to 1. * differs from vehicle only, $P<0.05$.

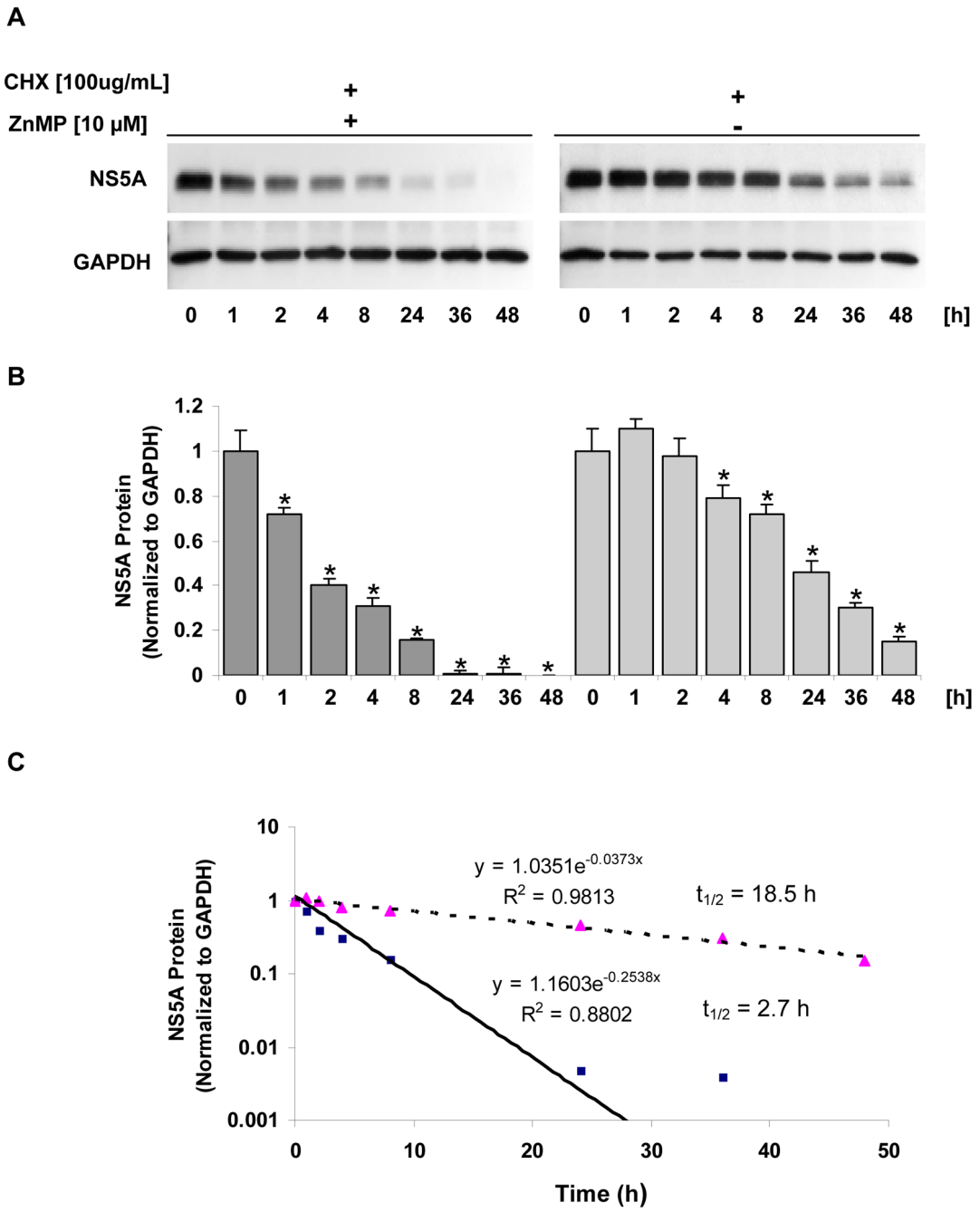


Figure 4. ZnMP-induced degradation of NS5A in 9–13 cells

(A) 9–13 cells were treated with 100 ug/mL cycloheximide (CHX) and with or without 10 μM ZnMP for the indicated periods (0, 1, 2, 4, 8, 24, 36, 48 h), and then cells were harvested and total proteins were isolated. NS5A or GAPDH protein levels were measured by Western blot. **(B)** The intensities of bands in panel A were quantified by densitometry. The band intensity of NS5A from sample (0 h) was set at 1. **(C)** Half-lives of NS5A protein in the absence (triangles) or presence (squares) of ZnMP.

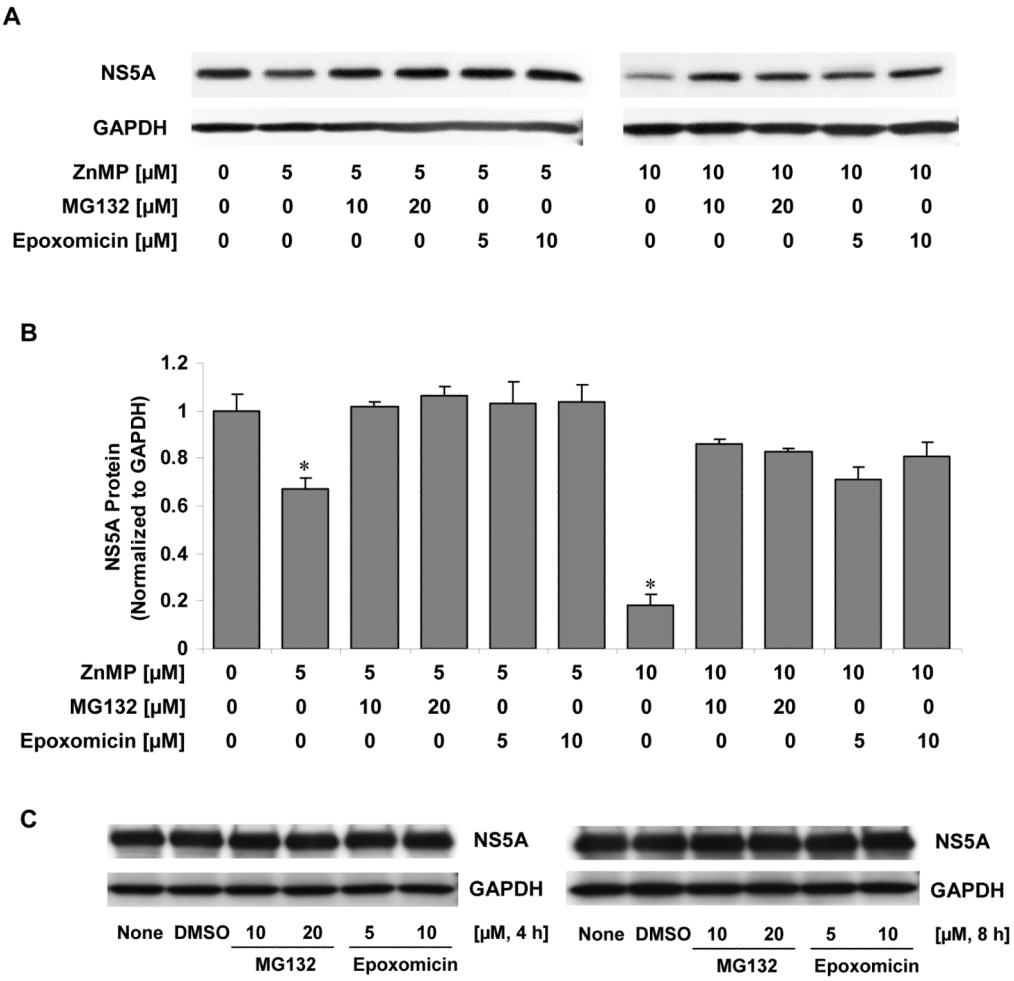


Figure 5. ZnMP down-regulates NS5A by a proteasome-dependent process

9–13 cells were treated with indicated concentrations of MG132 or epoxomicin 30 min before ZnMP treatment, the cells were subsequently exposed to ZnMP (5, 10 μM) or to vehicle alone (0 μM) as control for 4 h. NS5A and GAPDH protein levels were measured by Western blot. **(A)** NS5A protein levels in 9–13 cells treated with ZnMP (5, 10 μM) and different concentrations of epoxomicin (5, 10 μM) or MG132 (10, 20 μM). **(B)** The intensities of bands in panel A were quantified by densitometry. The amounts of NS5A protein were normalized to those for GAPDH, which did not vary with treatment. The band intensity of NS5A from vehicle alone was set equal to 1. * differs from vehicle only, $P<0.05$. **(C)** NS5A protein levels in 9–13 cells exposed to epoxomicin or MG132 in the absence of ZnMP.

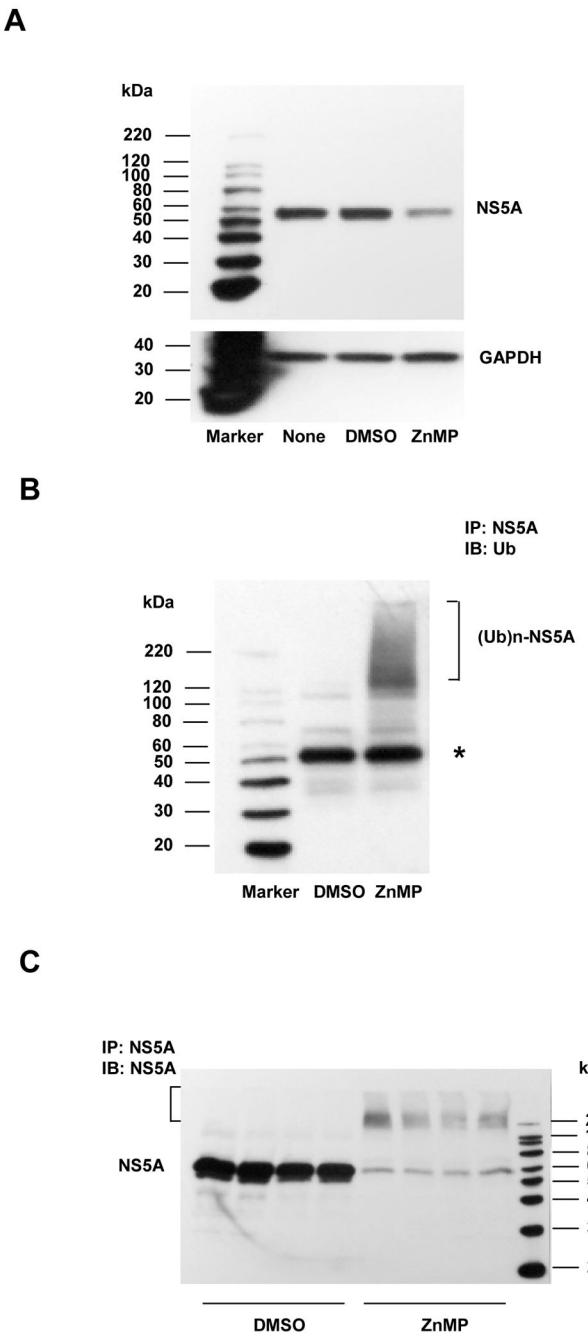
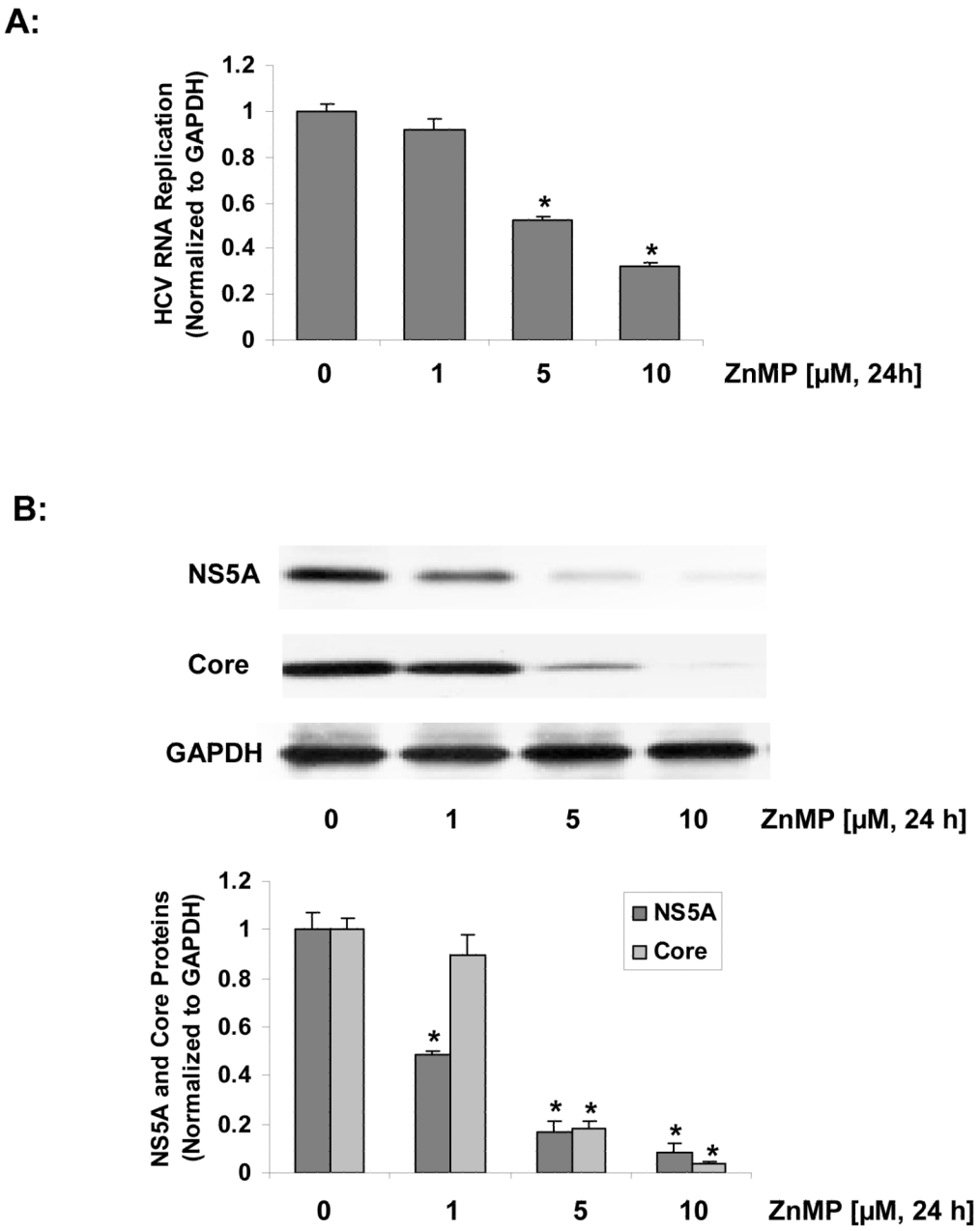


Figure 6. ZnMP-induced ubiquitination of NS5A

9–13 cells were treated without or with 10 μM ZnMP for 4 h. Total proteins were extracted for subsequent Western blot or immunoprecipitation analysis. Immunoprecipitation was carried out using anti-HCV NS5A antibody. Ubiquitin conjugation of NS5A [polyubiquitinated NS5A (Ub)n-NS5A] was examined with immunoprecipitation using an anti-HCV NS5A antibody and immunoblot using an anti-ubiquitin antibody. **(A)** Western blot analysis of NS5A protein levels before immunoprecipitation shows down-regulation of NS5A by ZnMP. **(B)** Ubiquitination of NS5A following ZnMP treatment, or vehicle (DMSO) only was compared. The positions of molecular mass markers (in kilodaltons) are indicated to the left of the gel. The bracket indicates polyubiquitinated NS5A. Asterisks

indicate cross-reacting immunoglobulin heavy chain. **(C)** Immunoblot analysis with an anti-NS5A antibody indicates that NS5A proteins were immunoprecipitated in panel B. The bracket indicates lower-mobility bands containing NS5A. These bands may represent polyubiquitinated NS5A.



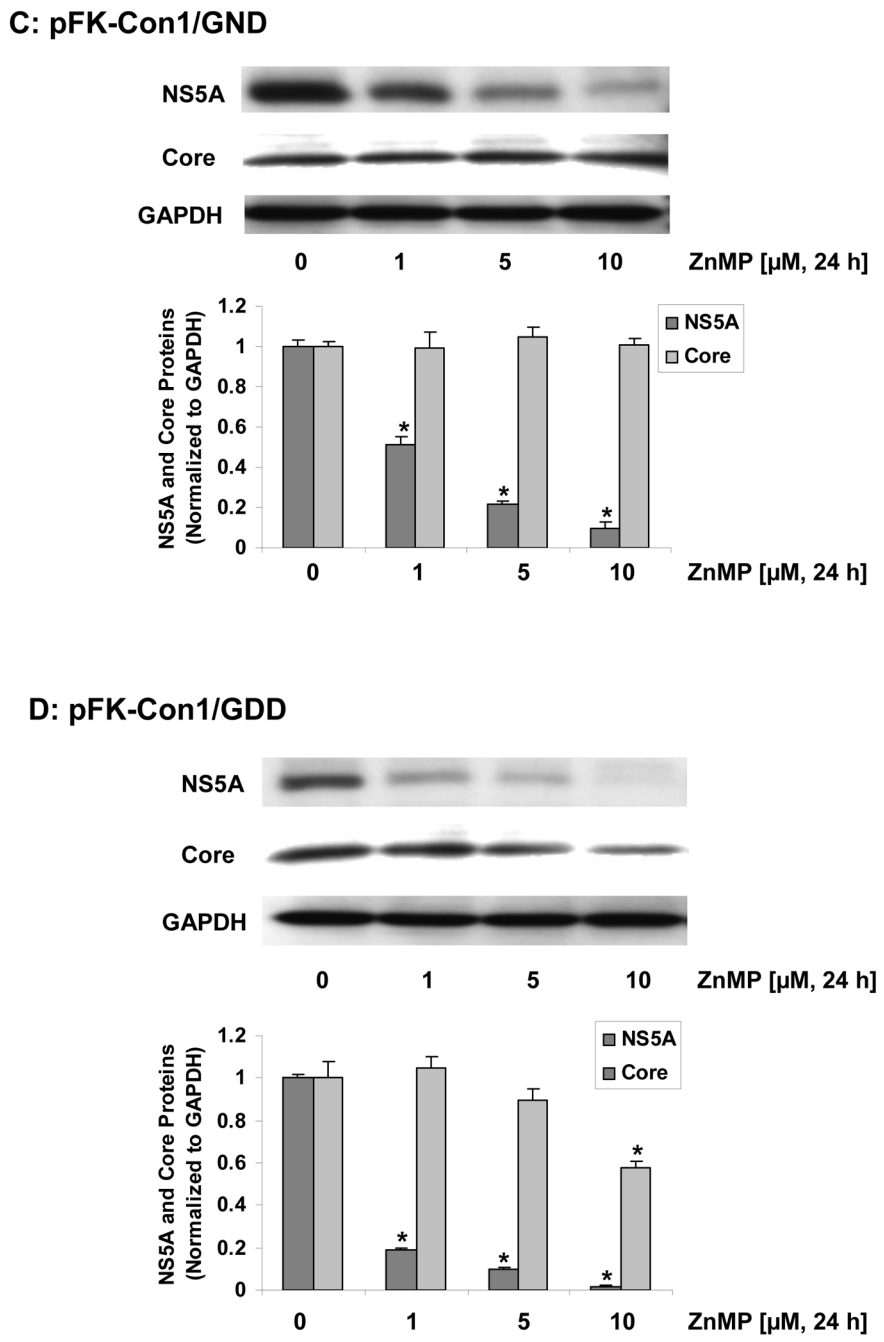
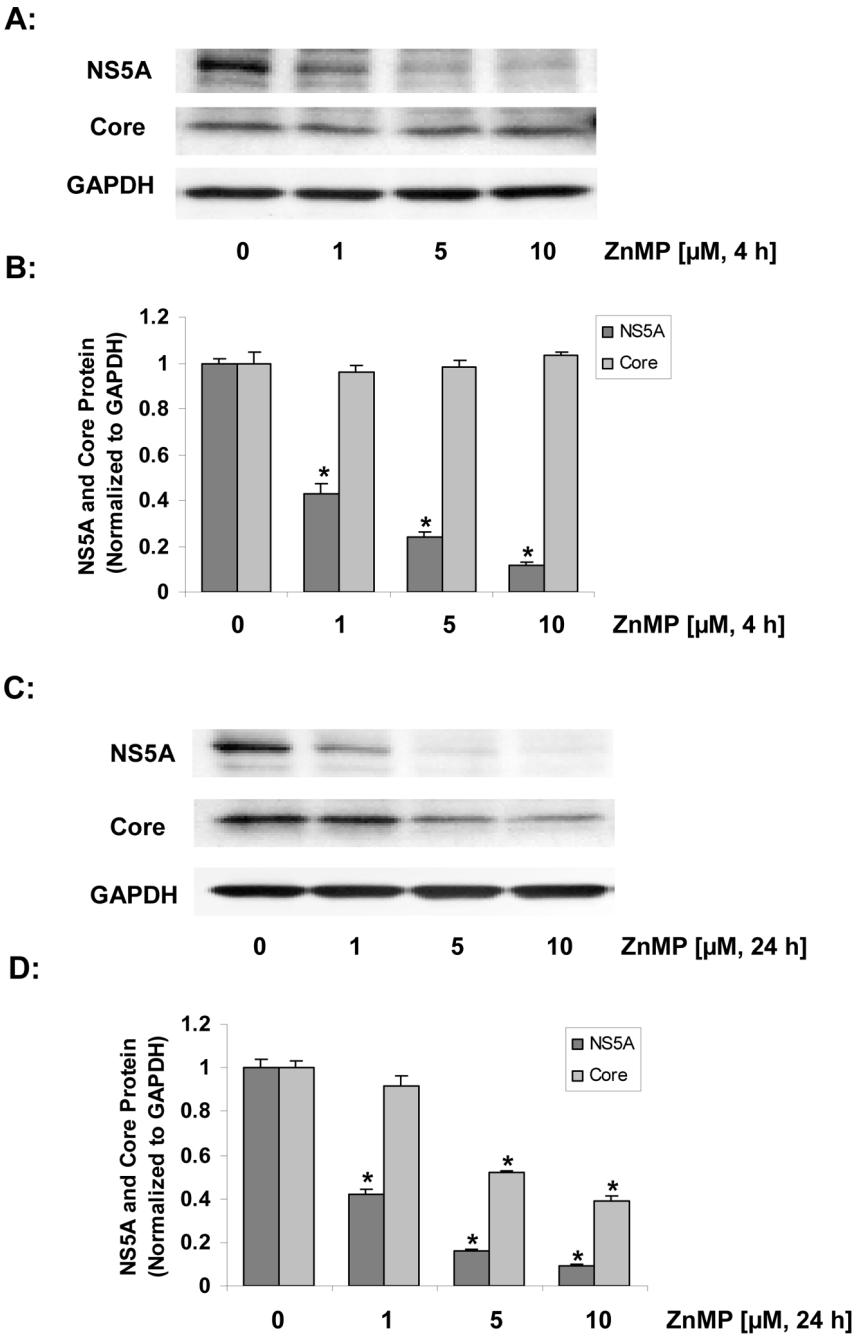


Figure 7. Down-regulation of HCV RNA and protein expression by ZnMP

Con1 cells were treated with indicated concentrations of ZnMP, or DMSO as control. After 24 h, cells were harvested and total RNA and proteins were extracted. HCV virus RNA was quantified by qRT-PCR, and the levels of HCV core, NS5A and GAPDH protein were measured by Western blots. Data are presented as means ± SE, n=3. * differs from vehicle only (ZnMP, 0 μM), P<0.05. **(A)** Effect of ZnMP on HCV RNA replication. **(B)** Effect of ZnMP on HCV core and NS5A protein levels. The upper panel shows photographs of representative Western blots. The bar graph shows quantitative results. **(C)** Down-regulation of HCV NS5A but not core by ZnMP in Huh-7/T7 cells transfected with pFK-Con1/GND. Huh-7/T7 cells were transfected with 0.8 ug/well of pFK-Con1/GND. 48 h after

transfection, cells were treated with ZnMP (0, 1, 5, 10 μM) for 24 h. Cells were harvested and total proteins were extracted for Western blots. **(D)** Effects of ZnMP on HCV core and NS5A proteins in Huh-7/T7 cells transfected with pFK-Con1/GDD. Huh-7/T7 cells were transfected with 0.8 ug/well of pFK-Con1/GDD. 48 h after transfection, cells were treated with ZnMP (0, 1, 5, 10 μM) for 24 h. The levels of HCV NS5A, core and GAPDH protein were measured by Western blots.



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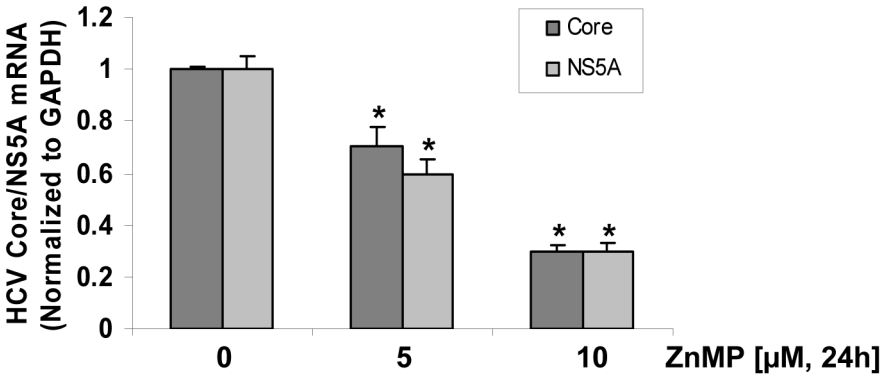

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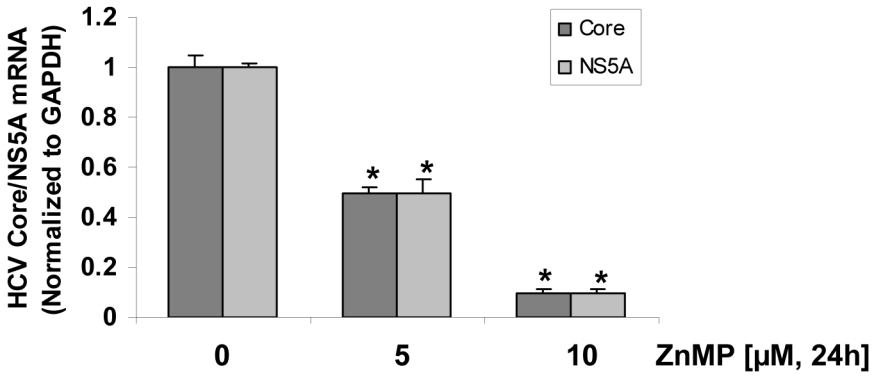

Figure 8. ZnMP down-regulates NS5A protein and display antiviral activity in the JFH1-based HCV cell culture system

Huh-7.5 cells were transfected with 2 μg/well of J6/JFH1 RNA by Lipofectamine 2000. After 48 h, cells were treated with indicated concentrations of ZnMP, or DMSO as control for 4 or 24 h. HCV RNA was quantified by qRT-PCR, and HCV core, NS5A and GAPDH protein levels were measured by Western blots. Data are presented as means ± SE, n=3. * differs from vehicle only (ZnMP, 0 μM), $P<0.05$. **(A)** Effects of ZnMP on NS5A and core protein levels following 4 h of ZnMP treatment. **(B)** The intensities of bands in panel A were quantified by densitometry. **(C)** Effects of ZnMP on NS5A and core protein levels following 24 h of ZnMP treatment. **(D)** The intensities of bands in panel C were quantified by densitometry. **(E)** Down-regulation of HCV RNA by ZnMP in Huh-7.5 cells transfected with J6/JFH1 RNA. **(F)** Effects of ZnMP on HCV RNA levels in Huh-7.5 cells infected with J6/JFH1 HCV. Huh-7.5 cells were infected with cell culture supernatants collected from the cells transfected with HCV RNA, and treated with ZnMP or DMSO as control for 48 h. HCV virus RNA was quantified by qRT-PCR, and the levels of HCV core, NS5A and GAPDH mRNA were measured by qRT-PCR.