Tizoxanide (2-hydroxy-N-(5-nitro-2-thiazoly1)benzamide) inhibits hepatitis C virus replication through modulation of NS5A hyperphosphorylation

A Dissertation
submitted to the Faculty of the
Graduate School of Arts and Sciences
Of Georgetown University
in partial fulfillment of the requirements for the
degree of
Doctor of Philosophy
in Microbiology

By

Abigail Montero, B.S.

Washington, D.C.
March 27, 2012
Copyright 2012 by Abigail Montero
All Rights Reserved
Nizoxanide (NTZ, Alinia®, Romark Laboratories, LC) is a licensed thiazolide anti-infective that is currently in clinical development for the treatment of chronic hepatitis C, but the antiviral mechanism has remained undetermined. The current investigations sought to determine the effect of tizoxanide (TIZ, active metabolite of NTZ) on intracellular HCV proteins to reveal a mechanism of action. The overall rate of reduction of HCV proteins in HCV replicon containing cell lines treated with TIZ was consistent with loss of viral RNA template. Examination of intracellular membrane preparations (where replication of HCV is localized) from HCV replicon cell lines revealed enhanced levels of hyperphosphorylated HCV NS5A (p58) peaking after 48 hours of TIZ treatment, coincident with the initial reduction of HCV RNA. The phosphorylation state of NS5A is established as a regulator of the switch from active viral genome replication to packaging and assembly; overproduction of p58 is established as being inhibitory to HCV replication. Consistent with this model, HCV RdRp activity on nascent HCV genomes in membrane preparations from TIZ-treated cultures was also reduced. Casein kinase I-alpha (CKIα) is a cellular kinase responsible for hyperphosphorylation of NS5A. CKI activity in intracellular membrane preparations from TIZ-treated HCV replicon cells was approximately 2-fold higher than in untreated cells in enzymatic assays. However,
TIZ had no direct effect on CK1α activity in enzymatic assays, including autophosphorylation, or on another cellular kinase associated with p58 formation, PLKI, in either cellular samples or direct enzymatic assays. **Conclusions:** These investigations provide a mechanism-of-action for the anti-HCV activity of TIZ. The primary cellular target for TIZ remains unidentified, but is most likely a protein involved in the upstream regulation of the cellular kinase, CK1α.
DEDICATION

First and foremost, this dissertation is dedicated to my husband José and my twins, Marco and Margaret. José, you are my love and my rock, but you are also my reality check and my drama filter. I know it hasn’t always been easy for you (or us), but I am so grateful you’ve been by my side, and that we have so many wonderful things to show for our hard work, especially our two beautiful, thriving, healthy children, who wouldn’t be what they are without you. For all these things and more, I thank you from the bottom of my heart. I love you, now and always. Marco and Maggie, your first 3 years have been amazing! You both have taught me many things, some simple and expected like patience and perseverance, and many things not so simple. You have taught me to laugh at the idiosyncrasies and unexpected joys of life. You have taught me the humor in silly phrases, and the importance of every single spoken word. You have shown me the joy in the little things in life, like stopping to put an earthworm back in the dirt, or catching snowflakes on our tongues. You have taught me to look at the bright side of things—a rainy day is never dreary when we have puddles to stomp in! You have taught me to tear down inhibitions and question convention—really, why can’t bowls be shoes? And why can’t the fingerpaints go on our shirts and not on the special fingerpainting paper? You have taught me to fight, and I mean really fight, for what I want. I have no reason to give up at anything when you two have overcome so many hurdles and exceeded all expectations. Through all the trials and triumphs of motherhood, some normal and some extraordinary and unique to the two of you, I have become a better wife, a better scientist, and a better person. But most importantly, you two have
taught me to love fiercely and unconditionally. I love you, my Bug and my Goose, and hope that some day I live up to my favorite name, “Mommy.”

Secondly, this dissertation is dedicated to my advisor, Dr. Brent Korba. Thank you for taking me on—I’m sure I wasn’t the easiest person to have as your first graduate student (and to all future Korba graduate students, I’m sorry—it’s probably my fault that he is the way that he is!). Thank you for allowing me to make my own way, even if it meant falling on my face. Thank you for giving me the space to grow as a person and as a scientist. Thank you for putting up with me, especially after the twins were born and my schedule became erratic (and sometimes non-existent). Thank you for teaching me how to think like a big kid, and for showing me how to love science with the joy of a little kid. But seriously, when are you going to get rid of the pipettor I broke—-5 YEARS AGO?!?! And for the final time, no, I did not spit in the cell cultures!

This dissertation is also dedicated to my beloved best friend Colleen Dutra. Even from one thousand miles away, you always know when I need you the most. Your unconditional love and support has gotten me through my toughest times. Thank you for all the tears you have dried, the hurts you have healed, the laughter we have shared, and the support we give each other. Thank you for always accepting me as I am and for reminding me of who I am. I love you Collyeen my Collyeen!!

This dissertation is also dedicated to my families. To my parents, your love and support has truly been unconditional. Thank you for believing in me even when I stopped believing in myself. Thank you for the foundation that taught me to love learning and that also taught me how to find my place in the world. I hope I am doing you justice by passing on the same foundation to my own children. Dad, I hope this
earns me a “That’ll do, Pig”, and Mom, your MOM degree will always be worth
infinitely more than my PhD. “I love you” and “thank you” never seem sufficient to
express all the love and deepest gratitude I have for you both, but here goes anyway: I
love you, and thank you….for everything! To my siblings, well, we never were good at
the touchy-feely thing, so we’ll just leave it at a simple, but very sincere, thank you and I
love you. To my in-laws, I forget I’ve only been part of the family for 5 years! You have
accepted me and loved me as your own from the very beginning. Such heartfelt
sentiments are never lost in translation. Muchas gracias y los quiero. Finally, to my
church family, for being there absolutely every time, through every trial and triumph,
every dirty diaper and desperate plea for sanity. I honestly would not have survived the
twins’ first year (or second or third…) without each and every one of you.

This dissertation is also dedicated to the members of my Georgetown family. To
the members of the Korba lab, past and present, for all their help both in the lab and out:
Dr. Changsuek Yon, Dr. Prasanth Viswanathan, Sunghae Uhm, Kristine Farrar, Karen
Gaye, and Sampa Mukherjee. Many of my protocols are working because of you! To
my thesis committee, whose tough love was exactly what I needed to keep fighting and
striving to be better: Dr. John Casey, Dr. Paul Cote, Dr. Radoslav Goldman, Dr. R. Pad
Padmanabhan, and Dr. Leonard Rosenthal. To the other faculty members of the
Microbiology department, who are always willing to answer even the most mundane
questions. To the other graduate students, past and present, for reminding me that there is
life outside of grad school, even if I’m only living it vicariously through you!

Finally, I thank God for the people in my life and for the ability to achieve this
goal. This one’s for You.
ACKNOWLEDGEMENTS

This work was supported by Romark Laboratories, LC and by NIAID contract NO1-AI-30046.

The Ava5 and Huh7.5 cell lines were supplied by Apath, Inc. The TIZ-9 cell line was supplied by J. Glenn (Stanford University Medical School, USA). The HCV-specific antibodies used for these experiments were provided R. Bartenschlager (University of Heidelberg, Germany).

Support and training were expertly provided by Dr. Brent Korba, Dr. Changsuek Yon, Dr. Prasanth Viswanathan, Sunghae Uhm, M.S., and Kristine Farrar, M.S.
# Table of Contents

Introduction .................................................................................................................................................. 1

The Hepatitis C Virus .................................................................................................................................. 2

Genome ....................................................................................................................................................... 2

Virus Attachment and Entry ....................................................................................................................... 2

RNA Translation and Polyprotein Processing ............................................................................................. 3

Viral Replicase Formation and RNA Replication ....................................................................................... 5

Viral Assembly ............................................................................................................................................... 7

NS5A-the Protein of Interest ..................................................................................................................... 8

HCV Cell Culture Models .......................................................................................................................... 12

Subgenomic Replicons .................................................................................................................................. 12

Full-Length and Infection Models .............................................................................................................. 14

Treatment of HCV Infections .................................................................................................................... 15

Nitazoxanide ............................................................................................................................................... 16

Experimental Goals ..................................................................................................................................... 20

Materials and Methods .............................................................................................................................. 22

Compounds ............................................................................................................................................... 22

Cell Culture Treatments ............................................................................................................................ 22

Cell Lysis .................................................................................................................................................... 24

Total Cell Lysis .......................................................................................................................................... 24

Intramembrane Preparations ...................................................................................................................... 24

Immunoprecipitation ............................................................................................................................... 25
Western Blotting ................................................................. 25
Antibodies ................................................................. 26
NS5B Polymerase Assay ............................................... 28
in vitro Kinase Assays .................................................. 28
  CKI ................................................................. 28
  CKII ............................................................... 29
  PLK1 .............................................................. 30
  PI4KIIIα ......................................................... 30
Statistical Analyses ..................................................... 31
Results ........................................................................... 32
  TIZ reduces NS protein levels in a manner consistent with loss of viral RNA ........................................... 32
  TIZ induces hyperphosphorylation of HCV NS5A in membrane preparations from replicon-containing cells ................................................................. 35
  RdRP activity on nascent viral replicon RNAs is downregulated in membrane preparations from TIZ-treated cells ................................................. 37
  TIZ enhances CKI activity in membrane preparations from replicon-containing cells, but not in in vitro assays ........................................... 40
  TIZ does not affect intracellular levels or processing of CKIα ................................................................. 42
  TIZ does not target other kinases implicated in NS5A phosphorylation or HCV replication ................................................................. 44
  Enhancement of eIF2α phosphorylation is not the primary antiviral mechanism of TIZ ................................................................. 46
Discussion ..................................................................... 52

x
LIST OF TABLES

Table 1. HCV therapies currently in clinical trials..................................................17
Table 2. Effect of TIZ on HCV RNA and NS proteins........................................34
Table 3. Effect of TIZ on HCV-associated cellular kinases.................................45
Table 4. Inducers of eIF2α phosphorylation do not affect HCV replication............50
LIST OF FIGURES

Figure 1. HCV genome and polyprotein processing.................................................4
Figure 2. Viral replicase formation and virion assembly............................................6
Figure 3. Structure of NS5A..................................................................................9
Figure 4. Structures of nitazoxanide and tizoxanide.................................................19
Figure 5. Schematic of the Con1 subgenomic replicon.............................................23
Figure 6. Specificity of anti-HCV NS protein antibodies..........................................27
Figure 7. Loss of HCV NS proteins correlates with loss of viral RNA.......................33
Figure 8. TIZ induces NS5A hyperphosphorylation...............................................36
Figure 9. TIZ reduces HCV NS5B RdRP activity in intracellular membrane preparations..........................................................38
Figure 10. TIZ increases CKI kinase activity in intracellular membrane preparations.........................................................41
Figure 11. TIZ does not affect CKIα autophosphorylation in vitro or distribution of intracellular CKIα splice variants.................................43
Figure 12. TIZ induces eIF2α phosphorylation.......................................................48
INTRODUCTION

Hepatitis C virus infections are a global burden, chronically infecting approximately 170 million people worldwide (1, 133); of these infections, 2.7 million occur in the United States (3). It is estimated that 350,000 deaths occur annually due to HCV-related liver diseases (1).

Acute infections are often asymptomatic and are spontaneously cleared within several weeks. Persistent, or chronic, infections are defined by detection of HCV RNA in the blood for 6 months or more after infection. It is estimated that up to 80% of all HCV infections progress to chronicity, causing fibrosis which ultimately leads to cirrhosis, end-stage liver disease, and/or hepatocellular carcinoma (HCC). HCV causes one-third of all HCC cases in the United States (3), and HCC is currently the third leading cause of cancer-related deaths worldwide (221).

There are six major genotypes of HCV, labeled 1 through 6, with nucleotide sequence divergence of 30-35% between each genotype. Most of the genetic differences lie in the envelope protein coding regions, whereas the core protein, the nonstructural proteins, and the 5’-UTR sequences are highly conserved. Genotypes are further categorized into subtypes (for example, genotypes 1a and 1b) with differences at 20-25% of nucleotide sites between subtypes, often occurring as phenotypically silent amino acid mutations. Each genotype is typically dominant in specific geographic areas. Genotype 1 is the most common genotype in North America (187).

Within an infected individual, HCV generates quasispecies, or a population of heterogeneous viral sequences produced from a single infecting sequence (134). These
changes are often generated by the poor fidelity of the viral RNA polymerase, and
contribute to host immunity evasion and antiviral resistance.

The Hepatitis C Virus

Genome

First cloned 20 years ago (32, 97), the hepatitis C virus is the sole member of the
Hepacivirus genus of the Flaviviridae family, which also includes dengue virus, yellow
fever virus, and the pestiviruses. It is an enveloped RNA virus with a positive-sense,
single-stranded genome that is 9.6kb in length. The RNA genome contains a single open
reading frame (ORF) encoding a 3,000-amino acid polyprotein precursor with
untranslated regions (UTRs) at both the 5’ and 3’ ends. The 5’-UTR contains the viral
internal ribosome entry site (IRES) (208), which is required for cap-independent
translation of the viral RNA (190), while the 3’-UTR is required for RNA replication (59,
103, 222).

Virus Attachment and Entry

HCV binds hepatocytes via the heterodimer formed by its two envelope
glycoproteins, E1 and E2 (34, 65). These proteins interact with glycosaminoglycans on
the host cell surface, and the viral particle is captured by the low-density lipoprotein
receptor (LDLr) (5, 140). The tight-junction proteins scavenger receptor BI (11, 181),
CD81 tetraspanin (11, 165), claudin-1 (52), and occludin (166) mediate HCV uptake in a
clathrin-dependent manner (16). Subsequent entry occurs through pH-dependent
endocytosis (206). Low endosomal pH disrupts the conformational arrangement of the
envelope proteins (112), causing disassembly of the viral coat and fusion of viral and endosomal membranes (16, 86, 106, 137, 206), and release of the viral RNA into the cytoplasm.

*RNA Translation and Polyprotein Processing*

Translation of the viral RNA occurs at the rough endoplasmic reticulum (ER). The tertiary structure of the viral RNA 5’-UTR undergoes changes that recruit the ribosome to the start site of translation (208, 212) and bind the translation initiation complexes with high affinity (190). Initiation occurs when the 40S ribosomal subunit binds directly to the IRES (94), and this complex subsequently binds the remaining host factors necessary for translation (152).

The resulting polyprotein is proteolytically cleaved into 4 structural and 6 nonstructural (NS) proteins (Figure 1) (71, 182). The structural proteins, including core (80, 83, 108), envelope protein 1 (E1) (83), envelope protein 2 (E2) (189), and an ion channel protein p7 (73, 118, 157), are generated by proteolytic activity of host signal peptidases at the core/E1, E1/E2, and E2/p7 junctions (150). The C-terminus of core protein is additionally processed by a host signal peptide peptidase (90), thus mobilizing core for translocation to lipid droplets (136, 149), where replication occurs.

The NS proteins are all membrane-associated (21, 22, 89, 93, 141) and include NS2, a cysteine protease (70, 84) that also plays a critical role in virion assembly (95, 131, 160, 167, 191); NS3, the viral serine protease (N-terminus) (43, 204) and helicase (C-terminus) (155, 193); NS4A, an essential cofactor for NS3 protease activity (53, 119); NS4B, which is required for replication complex formation (45) via heterotypic
Figure 1. HCV genome and polyprotein processing. Schematic representation of the viral genome. The 5’- and 3’- UTRs flank the coding region, which encodes a polyprotein that is cleaved by multiple proteases, generating 4 structural (C, E1, E2, p7) and 6 nonstructural (NS2, NS3, NS4A, NS4B, NS5A, NS5B) proteins. ▼, sites of host signal peptidase cleavage. ◀, NS2 protease cleavage site. ◀, NS3 protease cleavage sites. Figure adapted from (113).
interactions of its N- and C- termini (156), and for localization of the replication complex proteins (45, 49); NS5A, a multifunctional protein that exists in two phosphoforms, p56 and p58 (96, 196) (discussed below); and NS5B, the viral RNA-dependent RNA polymerase (RdRP) (12, 128). The NS2 cysteine protease cleaves the NS2/NS3 junction of the polyprotein, releasing the NS3-5B portion of the polypeptide. The NS3/4A serine protease cleaves the remaining junctions, sequentially releasing all other NS proteins (69). The NS proteins NS3/4A, 4B, 5A, and 5B are required for subsequent formation of the replicase complex and viral RNA replication (46, 139).

Viral Replicase Formation and RNA Replication

HCV RNA replication occurs in detergent-resistant membranous webs formed from ER membranes and lipid rafts (6, 45, 138, 139, 183). The membranous webs contain the viral replicase complex consisting of NS proteins and viral RNA (46, 142). The current model is incomplete, but offers a general picture of replicase formation (Figure 2). Membrane alterations are induced by NS4B, which independently associates with lipid rafts (45). NS5A p56 associates with the viral RNA (87), and both the NS5A-RNA complex and NS5B bind human vesicle-associated protein A (hVAP-A) independently (209), thus localizing NS5A, NS5B, and the viral RNA to the lipid rafts. NS3 is recruited to the replicase complex either independently (138) or by complexing with NS4A prior to joining the replicase complex (119). Besides its role as a critical cofactor for NS3 activity, NS4A is also necessary for later formation of NS5A p58 (96). The membranous web is stabilized by the presence of NS5A, as NS5A recruits the
**Figure 2. Viral replicase formation and virion assembly.** Replicase formation occurs at ER-associated membranes. *Upper panel* NS4B and the NS3/4A enzyme complex are recruited independently to the membrane structure. Both the NS5A-RNA complex and NS5B bind hVAP-A, then localize to the replicase complex. All NS viral proteins bind the RNA (black line). NS5A p56 recruits PI4KI1α for membrane complex integrity.

*Lower panel* Hyperphosphorylation of NS5A (denoted by black circle with P) by CKIα causes loss of association of NS5A with hVAP-A (and subsequent loss of RNA replication), and the NS5A-RNA complex is recruited to the lipid droplets to bind core during assembly of viral particles. NS2 binds the E1E2 heterodimer, p7, and the NS3/4A enzyme complex, localizing them to the assembly site as well. Figure adapted from (113). The black circle with P denotes general phosphorylation, not a specific number of phosphate groups.
cellular kinase PI4KIIIα to the replicase complex, thereby stimulating its kinase activity (14, 117, 173). Increased PI4KIIIα activity induces accumulation of its PI4P product, which directly contributes to the maintenance and integrity of the membranous web (13, 14, 117, 173, 192).

Once the replicase complex is formed in the membranous web, RNA synthesis is initiated de novo by highly structured RNA elements in the 3’-NTR of the template RNA strand and the polymerase activity of NS5B (130, 230). Multiple positive strand RNA products are synthesized from each negative strand replicative intermediate (12, 122, 128). The new RNA genomes are used for translation, further RNA replication, or are recruited for inclusion in infectious virions.

Viral Assembly

The switch from active RNA replication to assembly is believed to be regulated by NS5A hyperphosphorylation (Figure 2, (199)), although the details of the process remain unclear. CKIα kinase activity hyperphosphorylates NS5A p56 to generate p58 (171, 172), disrupting the interaction of NS5A with hVAP-A (51), causing dissociation of the NS5A-RNA complex with the replicase complex and ultimately preventing RNA replication (51). Assembly of new virions occurs in close proximity to core-enriched lipid droplets (LD) (19, 138). Nucleocapsid formation is triggered by interactions between core and NS5A-RNA (9, 135), allowing NS5A p58 to deliver the RNA to core protein. The NS2 protein acts as a scaffold to bring all other necessary components together for assembly. NS2 interacts with E1E2 heterodimers, p7 protein, and the NS3/4A enzyme complex (40, 95, 131, 160, 167, 191). The interactions with E1E2 induce localization of
NS2 to NS5A-rich membranes (95, 131, 167), and the hyperphosphorylated state of NS5A stabilizes NS2 within the assembly site (167). Assembled particles are transported through early to late endosomes to the host cell plasma membrane. The late endosome membranes fuse with the plasma membranes, and virions are released (109). The details of release are not well understood, although several host factors have been implicated, including claudin-1 (203), Vps4 (39), and the ESCRT-III complex (39).

**NS5A—the protein of interest**

The NS5A protein is of particular relevance for this dissertation and therefore necessitates further discussion. The NS5A protein is comprised of 3 domains, each with specific functions (Figure 3). Domain I is necessary for membrane association and replicase assembly (22, 47, 158, 201). Domain II is indispensable for RNA replication (200), and domain III is required for both RNA replication and virion assembly (9, 199, 200). Domain III also colocalizes NS5A with core protein on lipid droplets (9), a process that is critical for proper virion packaging. All 3 domains are capable of binding RNA (58, 87), and NS5A even interacts with the NS5B RdRP (184, 185), facilitating template selection (92) and RNA synthesis (169).

NS5A exists as two phosphoproteins, p56 and p58 (96, 196). The p56 phosphoform is generated by phosphorylation of serine residues between amino acids 2200-2250 and 2350-C-terminus (Figure 3) and is thought to exist as a heterogeneous population of proteins phosphorylated on different serine residues but migrating at similar molecular weights (196). NS5A p56 is required for several replicative functions,
Figure 3. Structure of NS5A. The NS5A protein is comprised of amino acids 1973-2419 of the polyprotein (based on JFH-1 sequence) as indicated in the figure, and is characterized by 3 domains, which are separated by low complexity sequences (LCS) of repetitive amino acid residues. Amino acids 2200-2250 and 2350-C-terminus contain serine residues that are basal phosphorylation sites (basal). The characterized hyperphosphorylation sites are denoted by ★ (serine residues 2197, 2201, and 2204). The N-terminus helix membrane anchor (helix) and the C-terminus CKII binding site (residues 2352-2419) are indicated. Figure adapted from (113).
including formation of the replicase complex on intracellular membranes (138); core-NS5A colocalization on lipid droplets (9); and interactions with NS5B and RNA (8, 17, 51, 87, 113, 184, 185), which are mediated by associations with hVAP-A (51, 64, 209). The hyperphosphorylated p58 phosphoform is generated by phosphorylation of p56 (146, 196, 199) by cellular kinase(s) at serine residues 2197, 2201, and 2204 (Figure 3), and is important for regulating the switch between viral RNA replication and packaging (51, 199). The proactive role of NS5A p58 in packaging, although not yet shown definitively, is supported by two major findings. First, high levels of hyperphosphorylation decrease RNA replication (8, 51, 147, 171). Domain III binds hVAP-A; NS5A hyperphosphorylation results in loss of interaction between NS5A and hVAP-A. NS5A then relocates the viral RNA away from the viral replicase complex, which ultimately results in reduction of RNA replication (51). Secondly, adaptive mutations that reduce p58 levels also reduce particle production (162).

Several kinases, identified by in vitro yeast kinase assays, have been implicated in phosphorylating NS5A, including mitogen-activated protein kinase kinase (MEK)-1, -6, and -7; AKT/protein kinase B; casein kinase II (CKII); and casein kinase I (CKI) (37). Inhibition of MEK-1/2 decreases HCV replication, which would predict increased NS5A p58 levels, but this inhibition did not lead to changes in NS5A phosphorylation (88). CKII has been shown to bind the C-terminus of native NS5A and phosphorylate it to the p56 isoform in vitro (101), but neither deletion of CKII phosphorylation sites in NS5A (87) nor small-molecule inhibition of CKII (88) significantly affect RNA replication in replicons. Studies of CKI have shown that overexpression of the alpha, delta, or epsilon isoforms all increase p58 levels in cultured cells; however, only siRNA directed against
CKIα inhibits formation of p58 (171). This inhibition is furthermore rescued by addition of exogenous CKIα (171). Later work showed that NS5A is indeed a direct substrate of CKIα (172).

CKIα is a member of the case kinase I family of serine/threonine kinases involved in many cellular processes including vesicular trafficking (74), cell cycle progression (75), p53-mediated DNA repair (102), and apoptosis (15). CKI kinases recognize substrates that have been previously phosphorylated by other kinases (57). CKIα is regulated in multiple ways, including the phosphorylation status of the substrate, and substrate access based on localization of CKIα and/or the substrate (57); modulation by phosphatidylinositol 4,5-bisphosphate (PIP2) (23); autophosphorylation of the C-terminal residues (26); and alternative splicing (72, 179, 197, 228). There are four splice variants of CKIα including CKIα, CKIαS, CKIαL, and CKIαLS (72, 179, 197, 228). CKIα and CKIαL are formed by splicing of the S insert (28). The splice variants have different cellular distributions and properties (28, 61, 228). The CKIαL isoform predominantly localizes to the nucleus (61); CKIα and CKIαS localize to membranes (61) and are most likely the isoforms that interact with HCV NS5A.

Recently, PLK1, another serine/threonine kinase (66, 77) involved in oncogenesis (44, 194), mitosis (50), DNA replication (207, 219, 224), and p53 regulation (7, 124), was implicated as an NS5A-associated kinase (31). HCV NS5A coimmunoprecipitates with PLK1, and the ratio of p58/p56 is lower in PLK1 knock-down cells and in PLK1 kinase inhibitor-treated cells (31). The conclusions drawn from the data, however, are inconsistent with established HCV biology. First, PLK1 is believed to phosphorylate NS5A to both the p56 and the p58 phosphoforms, which is biologically not advantageous
to the virus. If the same kinase is responsible for both forms, then the virus is unable to
regulate the levels of each isoform individually. NS5A p58 levels must be tightly
regulated to support sufficient RNA replication (p56) before the switch to packaging
occurs (p58). Secondly, NS5A p56 and p58 are both decreased in PLK1 knockdown
cells, and it is difficult to separate a clear decrease in one NS5A phosphoform compared
to the other from simple protein loss if the overall levels of the protein are decreasing.
For these reasons, CKIα is considered the primary kinase responsible for NS5A
hyperphosphorylation for the purposes of this dissertation.

**HCV Cell Culture Models**

*Subgenomic Replicons*

A major benchmark in HCV research was achieved with the creation of the first
cell culture replication system: the HCV subgenomic replicon, which produces NS
proteins NS3-NS5B, and forms the membrane-associated replicase complexes (67), but is
unable to package or infect due to the lack of structural genes. Using sequence from
virus isolated from a patient, Lohmann et al (129) synthesized a genotype 1b subgenomic
replicon containing the 5’ HCV IRES, a selectable neomycin resistance gene conferring
resistance to G418, the IRES from encephalomyocarditis virus (EMCV), HCV sequence
encoding the polyprotein from NS3 to NS5B, and the authentic HCV 3’ end. This
replicon replicated to modest levels in Huh7 cells, a hepatoma cell line, and was found to
sustain replication for more than one year (163). Blight et al (17) reported successful
replication of a genotype 1a subgenomic replicon in the same Huh7 cell line. Following
this work, Blight et al (18) reported enhanced RNA replication of a genotype 1b replicon (Con1) in Huh7.5 cells, a line of IFN-cured Huh7 cells (18).

Replication of the Con1 replicon can be enhanced in Huh7 cells. It is not fully understood how it occurs, but it is well established that specific adaptive mutations within the NS3-5B region are required to significantly increase RNA replication in Huh7 cells harboring subgenomic replicons. An adaptive S2204I mutation in NS5A is required for more efficient replication in Huh7 (17), but not Huh7.5 (18), cells. Other residues were later shown to be important as well, including S2197P in NS5A (196), and E1202G and T1280I in NS3 for both genotypes 1a (223) and 1b (107). Adaptive mutations have also been identified for NS4B and NS5B, but these do not significantly affect RNA replication (107) or, in the case of full-length models, assembly and infection (164).

The adaptive mutations in NS5A were, interestingly, found to affect either serine residues that are required for NS5A hyperphosphorylation (17, 127). These observations established for the first time that high levels of NS5A hyperphosphorylation are not essential for HCV replication in vitro. Indeed, replication increases when NS5A p58 levels are decreased (8), and replication decreases when p58 levels are increased (51, 147, 171). Regulation of adaptive mutations in NS5A is crucial for the virus, however, as the single S2204I mutation enhances replication, but a double A2199T/S2204I mutation decreases, and a triple S2197P/A2199T/S2204I mutation abolishes, RNA replication (18).

A JFH-1 (Japanese Fulminant Hepatitis) subgenomic replicon based on sequence isolated from a patient represents a third genotype, genotype 2a. This replicon is unique because it was shown to replicate efficiently in Huh7 cells without G418 selection and
without adaptive mutations (98). This replicon is comprised of T7 polymerase, the HCV 5’-UTR, the neomycin resistance gene, the EMCV IRES, the NS3-5B coding region, and the HCV 3’-UTR. This same replicon was later shown to replicate both in other hepatocyte (HepG2, IMY-N9) (41) and non-liver (HeLa, 293) cell lines (99).

*Full-length and Infection Models*

The first full-length genotype 1b genomic replicons were reported in 2002 (18, 91, 162), but the replicons were unable to produce infectious viral particles in Huh7 because of the adaptive mutations in NS3 and NS5A (164). Replication of a full-length genome was also achieved in Huh7.5 cells, but the ability to produce infectious virus was not determined (18).

In 2005, three groups independently created autonomously replicating, full-length, infectious HCV models using the JFH1 genome. Lindenbach and colleagues (121) constructed a full-length HCV genome by adding a core-NS2 region from either the J6 (genotype 2a) or the H77 (genotype 1a) genomes to the JFH1 subgenomic replicon (genotype 2a) and transfected the constructs into Huh7.5 cells. The other two groups (211, 229) *in-vitro* transcribed the entire viral genome from purified JFH1 plasmid DNA and electroporated the genome into Huh7 (211) or Huh7.5 (229) cells. All 3 groups showed high levels of viral replication with their constructs. Interestingly, of the two constructs created by Lindenbach *et al* (121), only the J6/JFH1 construct containing elements from only genotype 2a achieved efficient replication compared to the H77/JFH1 construct. Both Lindenbach *et al* (121) and Zhong *et al* (229) showed robust spread of infection throughout the original culture and when naïve cells were inoculated with
supernatant from the transfected culture, whereas Wakita et al (211) demonstrated only limited spread under both conditions. Importantly, all groups showed that these constructs are viable infection models for translational research, as replication was inhibited by treatment with IFN-α or relevant HCV-specific inhibitors in all cases.

Interestingly, the Wakita construct, which exhibits limited spread of infection in culture, replicates efficiently in chimpanzees (211), an observation that correlates with an earlier finding demonstrating the inverse relationship between efficient cell culture replication and infection in chimpanzees (27). This inverse relationship also illustrates the critical balance between replication and packaging that must be maintained for successful viral propagation. In cell culture, adaptive mutations reduce NS5A hyperphosphorylation, maintaining the virus in replication mode without proceeding to packaging. This is most likely why the full-length replicons reported in 2002 were not infectious, as they contained adaptive mutations (18, 91, 162) (164). The cell culture viruses that replicate in chimpanzees, however, contain no adaptive mutations and NS5A hyperphosphorylation occurs normally, initiating the switch to packaging and viral spread as the liver turns over.

**Treatment of HCV Infections**

Historically, standard of care for HCV infection was a combination therapy including pegylated interferon alpha (peginterferon) and ribavirin (60, 180). Peginterferon treatment is thought to work much like endogenous interferon-alpha (IFN-α) by stimulating a non-specific host antiviral response. The anti-HCV mechanism of ribavirin (RBV) is still unclear, but is thought to include mutagenesis of the viral RNA
and subsequent loss of viral fitness (38, 111, 231), and/or promotion of IFN signaling by induction of IFN-stimulated cytokines (54). However, less than half of patients infected with genotype 1, the most common genotype in North America, respond to this combination therapy (85), and side effects are common and severe (reviewed in (214)).

Recently, two direct acting antivirals (DAAs) were approved for use with peginterferon and ribavirin: boceprevir (Victrelis®) (10) and telaprevir (Incivek®) (227). Boceprevir reduces viral polyprotein processing while enhancing the host cell IFN response (132). Telaprevir binds to the viral NS3-4A protease, thereby inhibiting its enzymatic activity (120, 159). These compounds show increased response rates in the clinic in combination with peginterferon plus ribavirin, but confer additional side effects (10, 227), underscoring the continued need for additional therapies. There are numerous other therapies currently in clinical trials and include polymerase inhibitors, protease inhibitors, NS5A inhibitors, immunomodulators, and host target modulators. A representative list of these therapies is presented in Table 1, and includes those currently in phase 2 or phase 3 clinical trials (2, 205, 210, 217).

**Nitazoxanide**

One such new therapy is nitazoxanide. Nitazoxanide (NTZ, Alinia®, 2-(acetyloxy)-N-(5-nitro-2-thiazyl)benzamide, Romark Laboratories, LC) (Figure 4), a broad-spectrum thiazolide anti-infective, is licensed in the United States for the treatment of diarrhea caused by *Cryptosporidium parvum* and *Giardia lamblia* and is currently in development for the treatment of chronic HCV infection (175). Phase 2 clinical trials of
<table>
<thead>
<tr>
<th>Drug Name</th>
<th>Company</th>
<th>Clinical Phase</th>
<th>Description/Target</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NS5B Polymerase Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R7128</td>
<td>Roche</td>
<td>2</td>
<td>nucleoside</td>
</tr>
<tr>
<td>IDX-184</td>
<td>Idenix</td>
<td>2</td>
<td>nucleoside</td>
</tr>
<tr>
<td>RG7128</td>
<td>Roche</td>
<td>2</td>
<td>nucleoside</td>
</tr>
<tr>
<td>PSI-7977</td>
<td>Pharmasset</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>ABT-333</td>
<td>Abbott</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>ABT-072</td>
<td>Abbott</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>BI-207127</td>
<td>Boehringer Ingelheim</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>GS-5885</td>
<td>Gilead</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>RO 5024048</td>
<td>Roche</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>VX-222</td>
<td>Vertex</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>ANA 598</td>
<td>Anasys</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>BMS 791325</td>
<td>Bristol-Myers Squibb</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>VCH-759</td>
<td>ViroChem Pharma</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>Filibuvir</td>
<td>Pfizer</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>GS-9190</td>
<td>Gilead</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td>VCH 916</td>
<td>ViroChem Pharma</td>
<td>2</td>
<td>non-nucleoside</td>
</tr>
<tr>
<td><strong>NS3/4A Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMC435</td>
<td>Medivir/Tibotec</td>
<td>3</td>
<td>protease</td>
</tr>
<tr>
<td>BI-201335</td>
<td>Boehringer-Ingelheim</td>
<td>3</td>
<td>protease</td>
</tr>
<tr>
<td>ABT-450</td>
<td>Abbott</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td>BMS-650032</td>
<td>Bristol-Myers Squibb</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td>GS-9451</td>
<td>Gilead</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td>ITMN-191</td>
<td>Intermune</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td>Danoprevir</td>
<td>Roche</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td>Vaniprevir</td>
<td>Merck</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td>ACH-1625</td>
<td>Achillion</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td>GS-9256</td>
<td>Gilead</td>
<td>2</td>
<td>protease</td>
</tr>
<tr>
<td><strong>NS5A Inhibitors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMS-790052</td>
<td>Bristol-Myers Squibb</td>
<td>2</td>
<td>disrupts localization</td>
</tr>
<tr>
<td>AZD7295</td>
<td>AstraZeneca</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>New Interferons</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zalbin</td>
<td>Human Genome Sciences</td>
<td>3</td>
<td>albinterferon alfa-2b</td>
</tr>
<tr>
<td>Omega interferon</td>
<td>Intarcia</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Locteron</td>
<td>Biolex</td>
<td>2</td>
<td>gradual-release IFNα</td>
</tr>
<tr>
<td>PEG-rIL-29</td>
<td>Bristol-Myers Squibb</td>
<td>2</td>
<td>PEG-IFN λ</td>
</tr>
<tr>
<td>IET</td>
<td>Transition Therapeutics</td>
<td>2</td>
<td>interferon enhancer</td>
</tr>
<tr>
<td><strong>Immune Modulators</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drug Name</td>
<td>Company</td>
<td>Score</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------</td>
<td>-------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Zadaxin</td>
<td>SciClone Pharma</td>
<td>3</td>
<td>thymosin alpha 1</td>
</tr>
<tr>
<td>SCV-07</td>
<td>SciClone Pharma</td>
<td>2</td>
<td>STAT3 inhibitor</td>
</tr>
<tr>
<td>CYT107</td>
<td>Cytheris</td>
<td>2</td>
<td>IL-7</td>
</tr>
<tr>
<td>Suvus</td>
<td>Bioenvision</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Therapeutic Vaccines</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IC 41</td>
<td>Intercell/Novartis</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>GI 5005</td>
<td>Globeimmune</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>TG4040</td>
<td>Transgene</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Chronovac-C</td>
<td>ChronTech Pharma</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CSL 123</td>
<td>Chiron</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alisporivir</td>
<td>Novartis/Debiopharm</td>
<td>2</td>
<td>Cylophilin inhibitor</td>
</tr>
<tr>
<td>Nitazoxanide</td>
<td>Romark Laboratories</td>
<td>2</td>
<td>Phosphatidylserine-targeted antibody</td>
</tr>
<tr>
<td>Bavituximab</td>
<td>Peregrine Pharmaceuticals</td>
<td>2</td>
<td>miR-122 inhibitor</td>
</tr>
<tr>
<td>Miraviren</td>
<td>Santaris</td>
<td>2</td>
<td>α-glucosidase inhib</td>
</tr>
<tr>
<td>Celgosivir</td>
<td>Migenix</td>
<td>2</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MBL-HCV1</td>
<td>MassBiologics</td>
<td>2</td>
<td>polyclonal antibody</td>
</tr>
<tr>
<td>Civacir</td>
<td>NABI Biopharmaceuticals</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4. Structures of nitazoxanide and tizoxanide. Nitazoxanide is rapidly hydrolyzed to tizoxanide in cell culture or in the human gut by deacetylation. Images provided by Romark Laboratories.
NTZ in patients with chronic hepatitis C have shown enhanced efficacy when administered in combination with peginterferon or peginterferon plus ribavirin (176, 177). NTZ, and its active metabolite tizoxanide (TIZ, 2-hydroxy-N-(5-nitro-2-thiazoyl)benzamide) (Figure 4), exhibit potent antiviral activity against multiple genotypes of HCV, as well as replications carrying representative mutations that confer resistance to protease inhibitors and nucleosides in cell culture (105). Furthermore, NTZ and TIZ have been shown to act in a synergistic manner with clinically relevant DAAs (105). NTZ-11 and TIZ-9, HCV replicon-containing cell lines resistant to the antiviral effects of NTZ or TIZ, respectively, up to at least 50X the reported EC$_{50}$ (10µM), have been isolated (104), and the NTZ-resistance phenotype was shown to be conferred not by changes in the viral genome, but as a property of alterations in the host (104, 226). Furthermore, TIZ has been shown to have no direct inhibitory effect on the activity of HCV polymerase, protease, or helicase in enzymatic assays (226). However, the anti-HCV mechanism of TIZ is unknown.

**Experimental Goals**

It is of biologic interest to determine the mechanism of action of TIZ from both an antiviral view and with regard to what the mechanism can tell us about HCV-host interactions, assuming that TIZ targets a host process (discussed below). The primary goal of this dissertation was to determine the anti-HCV mechanism of TIZ. To determine if TIZ specifically targets one or more HCV NS proteins, we characterized expression levels of NS3, NS5A, and NS5B in both whole cell lysates and intramembrane preparations of Ava5 replicon-bearing cells treated with 1.0µM TIZ, and compared these
levels to those of control-treated cells. Given that TIZ does not directly inhibit HCV enzymatic activities (226), effects on the viral proteins may provide clues about the anti-HCV mechanism of TIZ. Possible outcomes of this analysis included a specific change in one or more HCV NS proteins, indicating a specific effect of TIZ on that (those) protein(s); or a general decrease in protein levels over time in relation to the viral RNA decrease, indicating that loss of protein correlates with loss of RNA.

Our secondary goal for this dissertation was to determine the host target of TIZ. We suspect a host target because TIZ is active against other viruses including hepatitis B virus, rotavirus, and influenza A virus (175), which target different host tissues and are structurally very different viruses; TIZ does not affect the in vitro enzymatic activities of NS3 protease, NS3 helicase, or NS5B polymerase (226); and resistance to TIZ is a property of the host cell, not the virus (104). It is probable that a single, or limited number of, host function(s) is targeted (e.g. a kinase or glucosidase). Because of the interrelated nature of the multiple host functions and signaling pathways, interference with even one function could result in multiple downstream effects. These effects would be dependent on the host environment induced by a specific virus, and the level of dependence of the virus on specific host processes.
**MATERIALS AND METHODS**

**Compounds**

Tizoxanide (TIZ, 2-hydroxy-N-(5-nitro-2-thiazolyl)benzamide) was provided by Romark Laboratories, LC (Tampa, FL). TIZ is soluble up to 10mM in DMSO (tissue culture grade, Sigma, St. Louis, MO), and was stable for up to two weeks at 4°C in amber vials. The average range of EC$_{50}$ for TIZ is 0.2-0.3µM; the EC$_{90}$ is 0.9-1.1µM (104, 105, 226). The anti-HCV nucleoside, 2’C-methylectyidine (2’CmeC) (143, 161) was purchased from Moravek Biochemicals (Brea, CA), dissolved in DMSO, and stored at -30°C.

**Cell Culture and Treatment**

Ava5 cells (Huh7.5 cells stably expressing a CON1, genotype 1b, subgenomic HCV replicon) (Apath, Inc., Brooklyn, NY) (17) (Figure 5) were grown in DMEM (4.5g/L glucose, without glutamine, without calcium; Quality Biologicals, Gaithersburg, MD) supplemented with 10% FBS (Atlas Biologicals, Fort Collins, CO), 1% glutamine (Quality Biologicals), 1% non-essential amino acids (Gibco, Carlsbad, CA), and 250µg/ml G418 (Geneticin, Gibco) as previously reported (105, 151). For the treatment studies, antiviral agents were replaced in fresh medium without G418 every 24 hours. TIZ-9 cells (TIZ-resistant RP7 cells (subgenomic replicon, genotype 1b, CON1)) (104) were cultured in the same manner as Ava5 cells, with the addition of 10µM TIZ to the medium during stock culture growth to maintain drug resistance. HCV RNA levels from cellular samples were analyzed using quantitative blot hybridization as previously described (105, 151).
Figure 5. Schematic of the Con1 subgenomic replicon. The replicon is comprised of the authentic 5’- and 3’- UTRs, the neomycin resistance gene for selection in cell culture (neo), the encephalomyocarditis virus IRES (E-I) to drive translation of the polyprotein, and the coding sequence for NS3-5B. Figure adapted from (17).
Cell Lysis

Total Cell Lysis

Ava5 cells grown in 6-well plates were rinsed once with 2ml ice-cold D-PBS, then lysed in 250µl RIPA buffer (Pierce, Rockford, IL) supplemented with 1X protease- and phosphatase- inhibitor cocktail (Pierce) per well. The lysates were collected and centrifuged at 1,000 x g for 15 minutes at 4°C, and the supernatant was stored at -70°C. Total protein concentration of each sample was determined using the BCA Protein Concentration Assay kit (Pierce) according to the manufacturer’s instructions.

Intramembrane Preparations

The intramembrane preparation (IMP) protocol was adapted from Hardy et al (82) and Aizaki et al (6). Each 100mm dish of Ava5 cells was rinsed twice with and scraped in 5ml ice-cold DPBS. For sufficient final sample concentration, two identically treated dishes were combined for each sample for a total of 10ml per sample. The samples were centrifuged at 900 x g for 10 minutes at 4°C. The supernatant was removed and the cell pellet was resuspended in 1ml hypotonic buffer (10mM Tris-HCl, pH 7.8, 10mM KCl, and 5mM MgCl₂), then incubated on ice for 15 minutes. The samples were homogenized by 50 strokes of a Dounce homogenizer with a tight-fitting pestle. The samples were centrifuged at 900 x g for 5 minutes at 4°C to pellet the cell nuclei, after which the supernatants were transferred to fresh tubes, and centrifuged at 15,000 x g for 20 minutes at 4°C to pellet the cellular membranes. The supernatants were removed, and the pellets were resuspended in 100µl storage buffer (hypotonic buffer + 15% glycerol). Samples were stored at -70°C for up to one month.
**Immunoprecipitation**

Fifty microliter aliquots of each IMP sample were incubated with 1µg anti-phosphoserine antibody (Invitrogen, Carlsbad, CA) for 1 hour at room temperature on a rocker. Twenty microliters of A/G PLUS agarose (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the samples and the reactions were incubated at 4°C for 24 hours on a rocker. The reactions were centrifuged at 500 x g for 5 minutes at 4°C and the supernatant was removed. The pellets were washed 3 x 1ml 1X PBS with centrifugation, then resuspended in either 40µl of 1X PBS for use in lambda phosphatase reactions, or 40µl of SDS sample buffer, boiled for 3 minutes, and subjected to SDS-PAGE.

Lambda phosphatase (λPP) reactions were performed according to manufacturer’s instructions (New England Biologicals, Ipswich, MA) using 20µl of IP sample, 400U (1µl) λPP enzyme, 1X MnCl₂, and 1X PMP buffer in each 50µl reaction. Reactions were incubated for 30 minutes at 30°C and stopped by addition of 25µl SDS sample buffer.

**Western Blotting**

Lysate or IMP samples (20µl) were mixed with 20µl 2X SDS sample buffer, boiled for 5 minutes, and separated by SDS-PAGE as indicated for each figure. The proteins were transferred to PVDF membranes using the iBlot™ gel transfer system (Invitrogen) and immunoblotted using the Snap i.d.™ protein detection system (Millipore, Billerica, MA) according to the manufacturer’s instructions. Briefly, membranes were blocked with 1X Blocker™ BSA (Pierce) in 1X TBS + 0.1% Tween-20 (TBS-T). After incubation with primary and secondary antibodies diluted in TBS-T, the membranes were incubated for 5 minutes at room temperature with approximately 6ml
ECL Plus chemiluminescence reagents (GE Healthcare, Piscataway, NJ) per miniblot. Chemiluminescent signals were detected by autoradiography with Amersham Hyperfilm™ ECL (GE Healthcare). To reprobe, membranes were placed in an appropriate volume of stripping buffer (2% SDS, 100mM β-mercaptoethanol, 62.5mM Tris, pH 6.8) and incubated at 50°C for 30 minutes, after which they were washed in TBS-T before immunoblotting. Films were scanned into a computer, and quantitation of immunoblot signals on film images was performed using ImageJ (4).

**Antibodies**

All primary antibodies were used at a 1:1,000 dilution in TBS-T unless otherwise indicated. The anti-eIF2α antibody was purchased from Cell Signaling Technology (Danvers, MA). The anti-phospho-eIF2α antibody was purchased from Enzo Life Sciences (Farmingdale, NY) and was used at a 1:500 dilution. Anti-HCV NS3, anti-HCV NS5A, and anti-HCV NS5B antibodies (Figure 6) were generous gifts of Dr. Ralf Bartenschlager (University of Heidelberg, Heidelberg, Germany). The anti-phosphoserine antibody was purchased from Invitrogen. The anti-CKIα antibody was purchased from Novus Biologicals (Littleton, CO) and was used at a 1:500 dilution. The anti-ADRP and anti-β-actin antibodies were purchased from AbCam (Cambridge, MA). Secondary HRP-conjugated antibodies, anti-rabbit and anti-mouse, were purchased from Millipore and used at a 1:10,000 or 1:5,000 dilutions, respectively.
Figure 6. Specificity of anti-HCV NS protein antibodies. Untreated lysates from either Huh7.5 (parental cell line) or Ava5 (replicon-containing cell line) cells were separated by SDS-PAGE and immunoblotted for the indicated NS protein. Molecular weight markers are indicated on the left, and arrows denoting the position of the indicated NS protein are indicated on the right of each panel. Cell lines are marked below each lane. β-actin is shown as a loading control.
NS5B Polymerase Assay

NS5B RdRP activity on nascent (resident) HCV genomes within IMPs was assessed in an *in vitro* assay constructed as previously described (170, 226), with modifications (assay optimized and performed by Dr. Changsuek Yon). Briefly, 20µl of membrane preparations were assessed in 50µl reactions containing 20mM Tris-HCl, pH 7.5, 10mM MgCl₂, 5mM DTT, 5mM KCl, 40ng/µl Actinomycin D, 1mM ATP, 1mM CTP, 5mM GTP, 10µM UTP, 20µCi α-³²P-UTP, 2.5mM phosphoenol pyruvate, 1U Pyruvate Kinase, and 1U RNAsin. Reactions were incubated at 35°C for 3 hours. HCV RNA products were purified using phenol and phenol/chloroform extraction and ethanol-precipitated at -70°C. RNA pellets were resuspended in 10µl of loading buffer and separated by electrophoresis using 0.8% formaldehyde agarose gels. Gels were dried, and radioactivity in each sample was quantitated using a Packard InstantImager β-Scanner.

*In vitro* Kinase Assays

*CKI*

CKI activity assays were performed according to manufacturer’s instructions for Casein Kinase I Assay Kit (Sigma) using 10µl IMP sample or 10µg whole cell lysate and 10µl of 1mg/ml CK I substrate (Millipore). The control reaction contained 20ng purified CKIα kinase (Invitrogen).

CKI inhibition assays were performed according to manufacturer’s instructions for Casein Kinase I Assay Kit (Sigma), with slight modifications. Experimental reactions contained 20ng purified CKIα (Invitrogen) or CKIδ (Sigma) kinase; 0.5µl of DMSO, 0.2µM TIZ, or 1.0µM TIZ, + 9.5µl dH₂O; 10µl of 1mg/ml CKI substrate (Millipore); and
10µl Assay Buffer for Casein Kinase Activity 5X. The control reaction contained no DMSO or TIZ. The reactions were allowed to proceed for 15 minutes at 37°C, after which 12µl ATP solution (1µl γ-32P-ATP (10mCi/ml: Perkin Elmer, Waltham, MA) for every 100µl dH2O) were added. The samples were incubated for an additional 15 minutes at 37°C. To stop the reaction, 38µl were spotted onto P81 cellulose phosphate paper, washed, and analyzed as for the CKI activity assays.

The CKIα autophosphorylation assay is based on Yokoyama et al (225). Ten nanograms of purified CKIα (Invitrogen) were combined with 10mM MgCl2, 40mM Tris-HCl, 2mM DTT, and 10µM γ-32P-ATP in 50µl reactions. The control reaction contained no DMSO or TIZ. The reactions were incubated at 30°C for 30 minutes, and stopped by the addition of 25µl of 2X SDS sample buffer. The products were purified by 12% SDS-PAGE and transferred to PVDF membranes. The incorporation of radioactive phosphates was detected by autoradiography.

CKII

CKII activity assays were performed according to manufacturer’s instructions for Casein Kinase II Assay Kit (Millipore). Experimental reactions contained 10µg total cell lysate + ADBI to 10µl. A blank reaction contained 30µl ADBI, and a positive control reaction contained 20µl ADBI and 20ng purified CKII enzyme (Millipore). CKII inhibition assays were performed as for CKII activity assays, except in the experimental reactions, the volume of ADBI was increased to 19.5µl, and the lysate samples were replaced by 0.5µl of DMSO, 0.2µM TIZ, or 1.0µM TIZ.
PLK1 assays were performed according to the manufacturer’s instructions for the PLK1 kinase enzyme system (Promega), with slight modifications. For assay of PLK1 activity within IMP samples, 20 µl of IMP sample were combined with 5 µg dephosphorylated casein substrate and 5 µl 250 µM $^{33}$P-ATP assay cocktail (150 µl 10 mM ATP, 100 µl $^{33}$P-ATP (1 mCi/100 µl), 5.75 ml kinase assay buffer I) in 25 µl reactions. Reactions were incubated at 30°C for 15 minutes, and 20 µl spotted onto P81 cellulose phosphate paper. Papers were washed 3 x 10 minutes in 1% phosphoric acid, added to 10 ml scintillation cocktail in a scintillation vial, and the radioactivity of each sample was determined using a Beckman scintillation counter.

PLK1 inhibition assays were performed as described for PLK1 activity assays by combining 100 ng purified PLK1 (Promega, Madison, WI); 5 µg dephosphorylated casein substrate; 5 µl $^{33}$P-ATP cocktail; DMSO, 0.2 µM TIZ, or 1.0 µM TIZ to a final DMSO volume of 1%; and water to a final reaction volume of 25 µl.

PI4KI$^{III}$$^{\alpha}$

PI4KI$^{III}$$^{\alpha}$ inhibition assays were performed by incubating 25 ng purified PI4KI$^{III}$$^{\alpha}$ (Millipore), 25 µM PI:PS lipid kinase substrate (Invitrogen), and either DMSO or 1.0 µM TIZ in 1X reaction buffer (4X reaction buffer (Millipore) diluted to 1X with dH$_2$O + 5 mM DTT) for 15 minutes. The reactions were activated by addition of 10 µl MgAc-ATP mix (6.25 µCi $^{33}$P-ATP + 25 mM MgAc in 1X assay buffer). After incubating 30 minutes at room temperature, the reactions were stopped by addition of 3 µl of 30% phosphoric acid and centrifuged at 16,000 x g for 10 minutes to pellet reaction products. The
products were washed 3 times in 200µl of 75mM phosphoric acid. The tubes were dried and placed inside scintillation vials with 10ml scintillation cocktail, and incorporated radioactivity was measured with a Beckman scintillation counter. Background control reactions consisted of complete reactions terminated by the addition of 30% phosphoric acid before activation with the MgAc-ATP mix.

**Statistical Analyses**

Data were analyzed for statistical significance using Prism 5.0d® (GraphPad Software).
RESULTS

TIZ reduces viral NS protein levels in a manner consistent with loss of viral RNA.

For all following experiments, we used TIZ, not NTZ. Although NTZ is hydrolyzed to TIZ in culture medium, we observed differences in metabolic efficiencies between different lots of NTZ, and, since NTZ is converted to TIZ in the gut prior to adsorption, only TIZ is presented to target cells in humans (175).

To determine the effect of TIZ treatment on HCV RNA, we treated Ava5 cells with 1.0μM TIZ (approximate EC$_{90}$) (105) for 24 to 72 hours, then extracted total RNA. HCV RNA was quantitated by blot hybridization as described in Materials and Methods. We found that treatment of Ava5 cells with 1.0μM TIZ induces a significant reduction in intracellular HCV RNA between 24 and 48 hours of treatment compared to control (Fig. 7A, Table 2).

We next wanted to characterize the effect of TIZ treatment on the viral NS proteins NS3, NS5A, and NS5B to determine whether protein expression decreases as a function of viral RNA loss. Although the Con1 replicon (17) used for these studies also expresses NS4A and NS4B, these proteins were not analyzed due to lack of suitable primary antibody. To analyze protein levels in replicon cells, Ava5 cells were treated with 1.0μM TIZ for 24 to 72 hours, then harvested for either lysate or intracellular membrane preparations (IMPs) to isolate viral replicase complexes (RCs), as described in Materials and Methods. It was important to analyze IMPs in addition to lysates because HCV replication is localized to lipid droplet structures associated with the ER, and 20-45% of HCV non-structural proteins are found in these regions (138). The lysate and
Figure 7. Loss of HCV NS protein levels correlates with loss of viral RNA.

(A) Ava5 cells were treated with 1.0µM TIZ for 24, 48, and 72 hours, harvested for RNA, and analyzed for HCV RNA by blot hybridization as described in Materials and Methods. Data are graphed as mean percent (± standard deviation) of RNA copies/cell compared to control at 24 hours (set as 100%) and are representative of triplicate samples. (B, C) Representative western blots of NS protein levels in lysate (B) and IMPs (C). Cells were treated for 24 to 72 hours with 1.0µM TIZ, harvested for lysate or IMP, and proteins were separated by SDS-PAGE followed by western blotting as described in Materials and Methods. β-actin (B) and ADRP, a marker for intracellular membranes (C), are used as loading controls. Length of treatment and concentration of TIZ are labeled above the gels, and proteins are indicated on the left. ** p < 0.0001 (t test).
### TABLE 2. Effect of TIZ on HCV RNA and NS proteins.

<table>
<thead>
<tr>
<th>% mean DMSO ± SD</th>
<th>DMSO</th>
<th>TIZ (1.0µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24hr</td>
<td>36hr</td>
</tr>
<tr>
<td>HCV RNA</td>
<td>100 ± 12</td>
<td>91 ± 9</td>
</tr>
<tr>
<td>Total lysate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS3</td>
<td>100 ± 5</td>
<td>103 ± 28</td>
</tr>
<tr>
<td>NS5A (p56)</td>
<td>100 ± 13</td>
<td>56 ± 13</td>
</tr>
<tr>
<td>NS5B</td>
<td>100 ± 5</td>
<td>71 ± 8</td>
</tr>
<tr>
<td>Membrane preps</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NS3</td>
<td>100 ± 28</td>
<td>70 ± 11</td>
</tr>
<tr>
<td>NS5A (p56 &amp; p58)</td>
<td>100 ± 16</td>
<td>71 ± 29</td>
</tr>
<tr>
<td>NS5B</td>
<td>100 ± 15</td>
<td>115 ± 46</td>
</tr>
</tbody>
</table>

The RNA and protein levels described for Figure 7 were quantitated and pooled from at least triplicate samples. Data are presented as a percentage of mean levels in control samples, set as 100% at each time point (± standard deviation (SD)). * $p < 0.02$ (t test). ** $p < 0.0001$ (t test).
IMP samples were separated by SDS-PAGE and immunoblotted for NS proteins.

Overall, protein levels were unchanged in TIZ-treated cells compared to control. Modest reductions in HCV nonstructural proteins in both cell lysates and IMPs were generally observed only after 72 hours of treatment (Fig. 7B, C respectively, and Table 2), consistent with the timing of reduction of viral RNA template by 48 hours (Fig. 7A, Table 2) and established viral protein half-lives of 12-15 hours (163). The single exception was significant reductions of NS5A as early as 36 hours in whole cell lysates (Table 2); the implications of this observation are discussed in the context of data presented in the next section.

**TIZ induces hyperphosphorylation of HCV NS5A in membrane preparations from replicon-containing cells.**

NS5A exists as two phosphoforms (96, 196). Only the p56 species was observed in the immunoblots of total cell lysates from the previous experiments; however, we were able to detect both p56 and hyperphosphorylated p58 NS5A in the IMP samples. To better analyze the effect of TIZ treatment on both phosphoforms of NS5A, IMPs from Ava5 cells treated with 1.0µM TIZ were first immunoprecipitated with an anti-phosphoserine antibody, then resolved by SDS-PAGE, and immunoblotted for NS5A. Samples were immunoprecipitated with anti-phosphoserine antibody because of limited availability of the NS5A-specific antibody. An aliquot of each IP product was also treated with lambda phosphatase (λPP) to confirm the slower migrating band as hyperphosphorylated NS5A, as p58, but not p56, is sensitive to this treatment (96, 196).

While the total amount of NS5A in the cellular membranes was essentially
Figure 8. TIZ induces NS5A hyperphosphorylation. Cell cultures were treated with 1.0μM TIZ for the indicated periods and harvested for IMPs as described in Materials and Methods. (A) Representative immunoblots of NS5A levels in IMPs of Ava5 cells treated with 1.0μM TIZ for 48 (left panel) or 72 (right panel) hours compared to control. (B) Ratio of NS5A p58/p56 following treatment. Values are presented as the mean ratio of treated samples relative to the mean ratio of each respective set of control (DMSO) cultures ± standard deviation. Data are representative of duplicate experiments, each with triplicate samples. (C) Representative immunoblot of immunoprecipitated NS5A before and after treatment with λPP, showing the change in migration pattern of NS5A p58. Both lanes are samples that were treated with 1.0μM TIZ for 72 hours before harvest for IMP. For (A, C), the p58 and p56 phosphoforms are indicated to the right of each image, and the treatment is indicated above each lane. * p < 0.002 (t test).
unchanged during most of the treatment period with TIZ compared to control (Table 2), exposure to 1.0µM TIZ increased the ratio of NS5A p58/p56 approximately 10- to 20-fold beginning at approximately 36 hours in membrane preparations from Ava5 cells, peaking after 48 hours, and remaining elevated for at least 72 hours of treatment (Figure 8B). Representative western blots of immunoprecipitated IMP samples from cells that were treated for 48 and 72 hours are shown in Figure 8A. The identity of the slower-migrating band was confirmed as NS5A p58 after incubation with λPP (Figure 8C), as it co-migrated with the p56 band after phosphatase treatment.

The effect of TIZ treatment on the ratio of NS5A p58/p56 was also examined in TIZ-9 cells. TIZ-9 cells are a line of RP7 replicon-containing cells that are resistant to treatment with up to 10µM TIZ (104), 10 times the reported EC₉₀ value (105). The relative levels of p56 and p58 are unchanged in TIZ-9 cells treated with 1.0µM TIZ for 72 hours compared to control (Figure 8B), indicating that the changes in NS5A phosphorylation in Ava5 cells after TIZ treatment are specific to the anti-HCV activity of TIZ.

**RdRP activity on nascent viral replicon RNAs is downregulated in membrane preparations from TIZ-treated cells.**

The data so far suggest that TIZ inhibits viral RNA replication by increasing levels of NS5A p58 without changing the overall levels of NS5A. According to the established HCV replication model (Figure 2), overproduction of p58 would induce significant disruptions of the association of the HCV RNA template with the replicase complex (8, 17, 51, 87, 113, 184, 185), ultimately inhibiting RNA replication, which has
Figure 9. TIZ reduces HCV NS5B RdRP activity in intracellular membrane preparations. Cultures were treated with the indicated concentrations of TIZ for 48 hrs, harvested for IMP, and HCV RdRP activity in aliquots of each IMP was assessed as described in Materials and Methods. (A) RNA products of RdRp reactions. Lane 1, DMSO control. Lane 2, IMP from control cultures treated with 1% NP40 (15 min) prior to RdRp reaction (negative assay control). Lane 3, 0.2µM TIZ. Lane 4, 1.0µM TIZ. Box indicates area of lanes quantitated for (B). (B) Radioactivity in each lane (areas indicated in (A)) was quantitated using a Packard InstantImager β-scanner. Values are presented as a percentage of mean radioactivity in control lanes (set as 100%) ± standard deviation and are compiled from triplicate experiments. *p < 0.001 (t test). Technical aspects of this experiment were conducted by Dr. Changsuek Yon and Sunghae Uhm, M.S.
been demonstrated in previous studies (51, 147). Since TIZ does not induce reductions in HCV proteins, especially NS5B, during the initial decline of HCV RNA levels (Table 1), it can be assumed that the number of HCV replicase complexes is not different from control-treated cells at this time. We predicted that in the presence of elevated levels of NS5A p58, NS5B RdRp activity on the resident HCV genomes would be proportionately reduced due to loss of association with RNA in the replicase complex. Loss of RNA synthesis would not be due to inhibition of NS5B RdRP activity, as TIZ does not inhibit this activity in vitro (226).

Ava5 cells were treated with either 0.2µM TIZ (approx. EC₅₀ (105)) or 1.0µM TIZ for 48 hours, harvested for IMPs, and were examined for relative RdRP activity on nascent HCV genomes using an in vitro polymerase assay as described in Materials and Methods. The 48-hour time point was chosen because there is no change in NS5B levels (Figure 7, Table 2), and it is the earliest time point at which HCV RNA replication is significantly decreased (Figure 7A).

Synthesis of nascent viral RNA was significantly reduced in IMPs from Ava5 cells treated with either 0.2µM TIZ (approx. 2-fold) or 1.0µM TIZ (approx. 8-fold) (Figure 9) compared to control. Reductions in activity were essentially equivalent to the overall reductions in HCV typically observed at these drug concentrations (approximately 2-fold and 10-fold, respectively). These results demonstrate a direct correlation of reduction in RdRP activity on nascent HCV genomes in the replication complex with a concomitant increase in NS5A p58 levels.
TIZ enhances CKI activity in membrane preparations from replicon-containing cells, but not in *in vitro* assays.

We hypothesized that induction of NS5A p58 by TIZ occurs through regulation of (a) host factor(s) responsible for NS5A hyperphosphorylation, since hyperphosphorylation does not occur as the result of a viral process but is dependent on the host. The cellular kinase casein kinase I, alpha isoform (CKIα), is one of the principle cellular kinases responsible for converting basally phosphorylated NS5A p56 to hyperphosphorylated p58 (171, 172). To determine if TIZ affects CKI activity, IMPs were isolated from Ava5 cells treated with 1.0µM TIZ for 24 to 72 hours, and CKI activity was assessed in these samples using a commercial enzymatic assay (Sigma) as described in Materials and Methods. Consistent with the increase in NS5A p58 at 48 hours (Figure 8), CKI activity was transiently, but significantly increased in IMPs after 48 hours of treatment with 1.0µM TIZ compared to control (Figure 10A). The enhancement of CKI activity in IMPs treated with TIZ was not sustained through 72 hours, however, possibly because of loss of NS5A p56 substrate observed in Figure 7 and described in Table 2.

The assay used to measure CKI activity in IMPs does not discriminate between different CKI isoforms. To determine if TIZ specifically up-regulates CKIα activity, purified CKIα enzyme (Invitrogen) was pre-incubated with different concentrations of TIZ, and kinase activity was assessed. As shown in Figure 10B, TIZ did not directly affect CKIα activity. To determine if TIZ requires interaction with other cellular
Figure 10. TIZ increases CKI kinase activity in intracellular membrane preparations. (A) CKI kinase activity in IMPs from Ava5 cells treated with 1.0µM TIZ for 24 to 72 hours was assessed in an *in vitro* assay as described in Materials and Methods. (B, C) Purified CKIα enzyme (Invitrogen) (B) or IMP from untreated cells (C) were pre-incubated with TIZ, and kinase activity was assessed using an *in vitro* CKI assay kit (Sigma) as described in Materials and Methods. Values are presented as mean percent radioactivity relative to the mean value for control (DMSO) cultures ± standard deviation. Data represent triplicate samples from two independent experiments. **p < 0.002 (t test).
components to interact with CKIα within the context of the membrane structure, IMPs from untreated cells were pre-incubated with TIZ, and CKI activity was assessed. TIZ did not enhance CKI activity in these samples (Figure 10C).

**TIZ does not affect intracellular levels or processing of CKIα.**

TIZ treatment could be indirectly enhancing CKIα activity in replicon-bearing cells by affecting regulation of the kinase, which is dependent on other cellular components and can occur before localization of CK1α to the membrane structure. The different splice variants exhibit different subcellular localizations and sometimes are recruited to other subcellular regions, and the level and efficiency of the kinase activity of each isoform is determined by the localization (28, 228). Activity of CKIα is also regulated primarily by autophosphorylation, alternative splicing, relative concentration, and the phosphorylation status of target substrates (26, 57, 228).

We first analyzed the effect of TIZ treatment on CKIα autophosphorylation using an *in vitro* assay based on a published protocol (225). Autophosphorylation of CKIα was not affected by TIZ (Figure 11A).

Next, we assessed the level and distribution of CKIα splice variants in lysates and IMPs of Ava5 and TIZ-9 cells treated with 1.0µM TIZ for 48 hours by western blotting. The 48-hour time point was chosen for analysis because that is when the highest CKI kinase activity was observed in Figure 10A. While different proportions of splice variants were detected in the IMPs compared to lysates (consistent with distributions established previously (28)), treatment with TIZ did not appear to affect the
Figure 11. TIZ does not affect CKIα autophosphorylation \textit{in vitro} or distribution of \textit{intracellular CKIα splice variants}. (A) An \textit{in vitro} CKIα autophosphorylation assay was performed as described in Materials and Methods. Molecular weight marker is indicated on the left, phosphorylated CKIα is indicated on the right (p-CKIα), and samples are indicated below the image. (B) Distribution of intracellular CKIα splice variants. Cultures were treated, total cell protein lysates or IMPs were harvested, and CKIα was analyzed by Western blot as described in Materials and Methods. Molecular weight markers are indicated on the left, and cell line and treatments are indicated above the image. Images are representative of three independent experiments.
relative distribution of CKIα splice variants within lysates compared to control, although
treatment with 1.0µM TIZ does appear to reduce overall CKIα levels in IMP samples
(Figure 11B). This observation is inconsistent with the increased CKI activity after 48
hours of treatment as shown in Figure 10A, but may support the decreased CKI activity
seen after 72 hours of treatment. The mechanism of enhancement of intracellular CKI
activity in TIZ-treated IMP samples cannot be explained at this time. It is possible that
TIZ alters an as-yet-unidentified protein or cofactor that regulates CKIα activity, but this
remains to be demonstrated.

**TIZ does not target other kinases implicated in NS5A phosphorylation or HCV
replication.**

Because CKIα is not the only kinase reported to phosphorylate NS5A, we also
analyzed the *in vitro* activity of other kinases reported to either phosphorylate or interact
with NS5A within the context of intracellular replicase complexes, using assays
described in Materials and Methods. Polo-like kinase 1 (PLK1) was shown to play a role
in hyperphosphorylation of NS5A in replicon-containing cell cultures (31). TIZ did not
affect the activity of this kinase either *in vitro* or in cellular lysates (Table 3). Another
CKI isoform, CKIδ, is also membrane-associated and, while shown to
hyperphosphorylate NS5A *in vitro*, intracellular siRNA silencing of this kinase does not
affect HCV replication or NS5A hyperphosphorylation (171). TIZ did not affect activity
of purified CKIδ *in vitro* (Table 3).

CKII has been shown to be responsible for basal phosphorylation of native NS5A
to form NS5A p56 (101). While TIZ treatment significantly decreased CKII activity in
**TABLE 3. Effect of TIZ on HCV-associated cellular kinases.**

<table>
<thead>
<tr>
<th>% mean DMSO ± SD</th>
<th>DMSO</th>
<th>1.0μM TIZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CKI, IMP</td>
<td>100 ± 7</td>
<td>164 ± 24*</td>
</tr>
<tr>
<td>CKIα, purified enz.</td>
<td>100 ± 4</td>
<td>99 ± 2</td>
</tr>
<tr>
<td>CKIδ, purified enz.</td>
<td>100 ± 20</td>
<td>106 ± 18</td>
</tr>
<tr>
<td>CKII</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cell lysates</td>
<td>100 ± 14</td>
<td>45 ± 12*</td>
</tr>
<tr>
<td>purified enz.</td>
<td>100 ± 11</td>
<td>68 ± 10</td>
</tr>
<tr>
<td>PLK1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IMP</td>
<td>100 ± 13</td>
<td>111 ± 29</td>
</tr>
<tr>
<td>purified enz.</td>
<td>100 ± 55</td>
<td>106 ± 51</td>
</tr>
<tr>
<td>PI4KIIIα, purified</td>
<td>100 ± 0</td>
<td>100 ± 18</td>
</tr>
</tbody>
</table>

CKIδ, CKII, PLK1, and PI4KIIIα were analyzed for kinase activity in either TIZ-treated lysates or IMP, or in the presence of TIZ *in vitro*, compared to DMSO as described in Materials and Methods. Data are presented as mean percent activity of treated samples compared to mean activity in control samples (set as 100%) ± standard deviation (SD).

* p < 0.02 (t test). IMP, intracellular membrane preparation. enz., enzyme.
cellular lysates, no such inhibition was observed in vitro (Table 3). Furthermore, a 3- to 4-fold reduction (p (t test) < 0.05) of CKII activity by TIZ treatment was also observed in samples from TIZ-9 cells (resistant to the antiviral effects of TIZ (104)) treated with 1.0μM TIZ compared to control TIZ-9 cells, suggesting that decreased CKII activity is a non-specific effect of TIZ treatment.

The membrane-associated kinase phosphatidylinositol 4-kinase III alpha (PI4KIIIα) has recently been shown to be essential for HCV-associated lipid droplet formation and maintenance (13, 14, 117, 173, 192), but does not affect NS5A phosphorylation (173). TIZ did not affect PI4KIIIα activity in vitro or in cell lysates (Table 3).

Enhancement of eIF2α phosphorylation is not the primary antiviral mechanism of TIZ.

Preliminary investigations into the anti-HCV mechanism of TIZ analyzed the effect of TIZ treatment on the expression levels of multiple host proteins involved in the unfolded protein response (UPR), a possible host target identified by initial screening performed by Romark Laboratories (unpublished observations). Data published by the Glenn lab at Stanford University confirmed the UPR as a possible target when they showed an increase in phosphorylation of both double-stranded RNA-dependent protein kinase (PKR) and of eukaryotic initiation factor 2, subunit alpha (eIF2α), in the presence of NTZ, the prodrug of TIZ, in vitro (48). PKR is phosphorylated in response to double-stranded RNA (115); one downstream effect of this phosphorylation is phosphorylation of eIF2α. The eIF2α protein is a critical component of the ternary initiation complex
required for translation of most mRNAs (reviewed in (154)), including the HCV polyprotein transcript. Phosphorylation of eIF2α inhibits translation (42, 114, 186) in response to ER stress. Although the presence of HCV subgenomic replicons induces ER stress, it decreases eIF2α phosphorylation (198). Thus, the data describing increased PKR phosphorylation and, in turn, increased eIF2α phosphorylation (48) imply that NTZ restores eIF2α phosphorylation, which results in decreased HCV polyprotein translation.

We then sought to further characterize eIF2α phosphorylation as a mechanism of TIZ. Lysates were harvested from Huh7.5 and Ava5 cells treated with 1.0μM TIZ for 72 hours, separated by SDS-PAGE, and immunoblotted for total eIF2α and phosphorylated eIF2α (phospho-eIF2α). We found that while total eIF2α levels are only modestly altered by TIZ, treatment of Ava5 cells with 1.0μM TIZ for 72 hours induces higher levels of phospho-eIF2α compared to control (Figure 12, compare lanes 3 and 4), consistent with published observations using NTZ treatment (48). HCV replication in Ava5 replicon-bearing cells lowers the levels of phospho-eIF2α compared to parental Huh7.5 cells (Figure 12, compare lanes 1 and 3), consistent with established literature (198), and treatment with TIZ appeared to restore the levels of phospho-eIF2α in Ava5 cells to those found in the parental Huh7.5 cells (Figure 12, compare lanes 4 and 1). Furthermore, increased phospho-eIF2α after TIZ treatment is an effect specific to the presence of the virus, as the effect was not observed in Huh7.5 cells treated with TIZ (Figure 12, compare lanes 1 and 2). Importantly, the increase in eIF2α phosphorylation does appear to be associated with the antiviral activity of TIZ because the effect was not observed after TIZ treatment of TIZ-resistant TIZ-9 cells (Figure 12, compare lanes 5 and 6).
Figure 12. TIZ induces eIF2α phosphorylation. Huh7.5 and Ava5 cultures were treated for 72 hours with the indicated compounds, harvested for cell lysate, separated by SDS-PAGE, and eIF2α levels were analyzed by western blotting as described in Materials and Methods. Treatment, cell lines, and the ratio of phospho-eIF2α/total eIF2α (P/UP) for each sample are displayed above the gel. A molecular weight marker is indicated to the left. Proteins are identified to the right. Image is representative of triplicate experiments.
If the primary antiviral mechanism of TIZ is through regulation of eIF2α phosphorylation, we reasoned that other compounds inducing phosphorylation of this protein should also be able to inhibit HCV replication. Therefore, we assessed the ability of a number of compounds, known to increase levels of phosphorylated eIF2α by different mechanisms, to inhibit HCV replication in Ava5 cells. Ava5 cells were treated for 72 hours with DTT, a strong activator of PERK (116) (PKR-like ER kinase), a UPR-specific kinase that is phosphorylated in response to general ER stress (81) and in turn phosphorylates eIF2α (81, 188); salubrinal, which inhibits the dephosphorylation of eIF2α (20); thapsigargin, a strong inducer of ER stress (202) and inhibitor of protein translation initiation (218) through eIF2α phosphorylation (33, 76); and sodium arsenite, which induces eIF2α phosphorylation without activating PERK or PKR (24). We also treated Ava5 cells with cycloheximide, a compound that inhibits general translation (25, 148). A dose three-fold lower than the highest non-cytotoxic dose, as determined by preliminary neutral red cytotoxicity assay, was used for each compound. After treatment, cells were harvested for total RNA, and HCV RNA was quantitated by blot hybridization as described in Materials and Methods.

Increased phospho-eIF2α levels after treatment with the various inducers was confirmed for each compound by western blotting (Table 4). None of the compounds was able to inhibit HCV RNA replication below a cytotoxic dose (Table 4), demonstrating that up-regulation of phospho-eIF2α by itself is not sufficient to inhibit HCV replication.
Table 4. Inducers of eIF2α phosphorylation do not affect HCV replication.

<table>
<thead>
<tr>
<th>Inducer</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Treatment Dose</th>
<th>% increase, phospho-eIF2α</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT (mM)</td>
<td>2.1</td>
<td>&gt; 2.1</td>
<td>1.4</td>
<td>180</td>
</tr>
<tr>
<td>Salubrinal (µg/ml)</td>
<td>10</td>
<td>&gt; 10</td>
<td>7</td>
<td>188</td>
</tr>
<tr>
<td>Thapsigargin (µg/ml)</td>
<td>0.01</td>
<td>&gt; 0.01</td>
<td>0.007</td>
<td>170</td>
</tr>
<tr>
<td>Sodium Arsenite (µM)</td>
<td>0.002</td>
<td>&gt; 0.002</td>
<td>0.001</td>
<td>165</td>
</tr>
<tr>
<td>Cycloheximide (µM)</td>
<td>1.2</td>
<td>&gt; 1.2</td>
<td>0.8</td>
<td>n.d.</td>
</tr>
<tr>
<td>Tunicamycin (µM)</td>
<td>2.5</td>
<td>&gt; 2.5</td>
<td>1.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>Geldanamycin (µM)</td>
<td>2.9</td>
<td>&gt; 2.9</td>
<td>1.9</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sialic acid (mM)</td>
<td>43</td>
<td>&gt; 43</td>
<td>28.7</td>
<td>n.d.</td>
</tr>
<tr>
<td>Brefeldin A (µg/ml)</td>
<td>0.01</td>
<td>&gt; 0.01</td>
<td>0.007</td>
<td>n.d.</td>
</tr>
<tr>
<td>Calyculin A (µg/ml)</td>
<td>0.13</td>
<td>&gt; 0.13</td>
<td>0.09</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Ava5 cells were treated for 72 hours with the indicated compounds, and CC<sub>50</sub> (concentration at which the compound is cytotoxic to 50% of cells) values were determined as described in Materials and Methods. Ava5 cells were treated for 72 hours with each compound at a three-fold lower dose than the respective CC<sub>50</sub> (treatment dose), and then HCV RNA levels were assessed and EC<sub>50</sub> (concentration at which 50% of viral RNA levels are inhibited) values were calculated for each compound as described in Materials and Methods. Phospho-eIF2α levels were quantitated from western blots of Ava5 lysates treated for 72 hours with the indicated treatment dose of each compound and are presented as a percent increase in protein level compared to control level (set as 100%). All values are calculated based on triplicate samples. n.d., not determined.
Since the presence of HCV subgenomic replicons inhibits eIF2α phosphorylation (Figure 12 and (198)), we next hypothesized that loss of virus after TIZ treatment reverses the HCV-induced repression of eIF2α phosphorylation. To test this hypothesis, we treated Ava5 cells with up to 30µM 2’CmeC, a polymerase nucleoside inhibitor (143, 161), conditions under which HCV is inhibited to the same or greater degree as 1.0µM TIZ (105). Treatment of Ava5 cells with up to 30µM 2’CmeC (10X the EC₉₀) did not alter levels of phospho-eIF2α, demonstrating that loss of viral RNA to the same or greater level as observed with TIZ treatment is not sufficient to immediately restore the levels of phosphorylated eIF2α (Figure 12, lanes 7-10). The mechanism by which TIZ induces phospho-eIF2α, and the significance of this observation relative to the antiviral mechanism of TIZ, remain unclear at this time.
DISCUSSION

In these studies, we have provided evidence that TIZ inhibits HCV replication primarily by increasing NS5A p58 levels, while concomitantly decreasing p56 levels, in HCV replication complexes. This is supported by a lack of an effect by TIZ on p58 levels in a TIZ-resistant cell line (TIZ-9), and a reduction of synthesis of nascent viral RNA in intracellular HCV replicase complexes by HCV polymerase. Overall, the kinetics of loss of HCV proteins under TIZ treatment are consistent with a loss of RNA template and previously described protein half-lives (12-15 hours) (163). TIZ is inactive against HCV polymerase, protease, and helicase activities in enzymatic assays (226).

This antiviral mechanism is consistent with the current model for regulation of HCV replication and virus formation by NS5A. The p56 phosphoform of NS5A allows for the association of the viral RNA with NS5B, the viral RNA-dependent RNA polymerase (87, 184, 185), via hVAP-A interactions (51, 64, 138, 209), and also promotes recruitment of the other viral components to the viral replicase complex, facilitating RNA replication. When NS5A is hyperphosphorylated to p58, the association of NS5A with hVAP-A and, therefore, that of the HCV genome with the viral replicase complex, are lost (51), and association of the viral genome with the packaging complex is favored (51, 199). Overproduction of p58 would relocalize the HCV genomes away from the viral replicase complex and have the net effect of lowering polymerase activity due to fewer genomes being present in the NS5B-containing replicase complexes. Our observation of a loss of NS5B RdRP activity on nascent HCV genomes in replication
complexes in IMPs containing elevated levels of NS5A p58 provides a direct test of this established model.

The membrane-associated cellular kinase CKIα has been shown to be responsible for NS5A hyperphosphorylation in cell culture (171, 172). Elevated levels of p58 induced by overproduction of CKIα have been previously shown to down-regulate HCV replication (171, 172). Consistent with these observations, we found a transient increase in CKI activity in IMPs from TIZ-treated HCV replicon-bearing cells (Figure 10A), correlating temporally with elevations in NS5A p58 levels (Figure 8A). CKIα activity in the drug-resistant cell line (TIZ-9) was unaffected by TIZ treatment. Unexpectedly, TIZ did not directly affect CKIα activity in vitro (Figure 10B, C), nor did it significantly affect the intracellular levels or distribution of CKIα splice variants (Figure 11), suggesting that TIZ may affect an upstream cellular factor responsible for regulation of CKIα that remains to be identified.

Increased CKI activity as a result of TIZ treatment does not exclude an effect on other kinases known to phosphorylate NS5A. However, no change in activity was observed in the presence of TIZ for two other cellular kinases capable of hyperphosphorylating NS5A, PLK1 (31) and CKIδ (171) (Table 3). CKII activity, which regulates NS5A p56 levels (101, 171), was significantly inhibited by TIZ treatment both in cell culture and in an in vitro assay (Table 3). However, the decrease in CKII activity in the presence of TIZ appears to be most likely unrelated to the antiviral mechanism of TIZ for three reasons. First, CKII is responsible for basal phosphorylation of NS5A to yield the p56 phosphoform, not p58 (101, 171). Secondly, neither deletions of CKII phosphorylation sites in NS5A (87), nor small-molecule inhibitors of CKII (88),
significantly affect HCV replication in cell culture. Finally, we found that CKII activity was decreased to the same degree in drug-resistant TIZ-9 cells following exposure to TIZ.

The broad spectrum of antiviral activities of TIZ (175), the host-related patterns of HCV resistance to TIZ (226), and the lack of a direct activity of TIZ on HCV enzymatic activities (226) strongly indicate a cellular target. We have shown increased eIF2α phosphorylation in Ava5 cells treated with TIZ, and Elazar et al (48) have also reported increased eIF2α phosphorylation and increased autophosphorylation of PKR in replicon cells treated with NTZ, the prodrug for TIZ. We have further demonstrated that increased eIF2α is not sufficient, by itself, to inhibit HCV replication. Compounds that are known to increase eIF2α phosphorylation by several different mechanisms did not exhibit anti-HCV activity. The reversal of HCV-related suppression of eIF2α phosphorylation to levels found in the parental cell line is correlated to TIZ antiviral activity since it was not observed in drug-resistant TIZ-9 cells. The increase in eIF2α phosphorylation was not simply an immediate consequence of reduced HCV replication since treatment with a DAA nucleoside inhibitor, 2’CmeC, did not result in a restoration of eIF2α phosphorylation. This observation also appears to be specific to HCV since TIZ treatment of the non-HCV carrying parental cell line did not alter phospho-eIF2α levels, and previously reported TIZ treatment studies of influenza virus and rotavirus did not show enhancement of eIF2α phosphorylation (178).

eIF2α serves as a translation initiation factor for transcripts with a 5’ cap, which the HCV transcript does not have. Therefore, the increase in phospho-eIF2α observed after TIZ treatment is likely an indication of modulation of a different component of the
signaling pathway by TIZ and not a direct effect on HCV replication through decreased protein translation. It is possible that the increase in phospho-eIF2α may be a result of a TIZ-induced reduction of functional NS5A p56. NS5A has been shown to suppress the host cell IFN signaling pathway (for review, see (195)). In particular, NS5A binds PKR, a key regulator of eIF2α phosphorylation, thereby inhibiting dimerization and downstream signaling (62, 63). The data presented here showing a decrease in cytoplasmic NS5A levels suggest that less PKR is bound by NS5A, which may in turn restore PKR signaling, including eIF2α phosphorylation. The increase in in vitro PKR autophosphorylation in the presence of TIZ reported by Elazar et al (48) supports this theory. The combined data do not exclude the possibility of a role for a TIZ-related relief of repression of host response pathways as a secondary contribution to the antiviral effect of this drug.

The efficacy of a drug targeting a host protein critically involved in HCV replicative functions has been demonstrated with cyclophilin inhibitors. Cyclophilins (CyP) are members of the immunophilin peptidyl-prolyl cis-trans isomerase family, and also function as molecular chaperones (213). Cyclophilins play an essential role in HCV replication by acting as molecular chaperones for both NS5A and NS5B (29, 220). The cyclophilin inhibitor cyclosporin A (CsA) effectively and specifically inhibits HCV replication in both replicon cells and HCV-infected hepatocytes (144, 145, 215). CsA competes with NS5A for binding to cyclophilin A (CyPA) (78, 79), thereby blocking the association of NS5A with the isomerase pocket of CyPA (30) and preventing the proper folding of NS5A (29). CsA treatment also prevents association of NS5B with the replicase complex (126) and abolishes the RNA binding affinity of NS5B (125, 216).
Resistance to CsA treatment can be generated in culture, is mediated by CyPA (220), and results in mutations in NS5A (30, 35, 55, 68, 168) and NS5B (55, 125, 174). The mutations in both proteins are believed to change the conformation of the protein so that the isomerase activity of CyPA is no longer needed (35, 55, 125, 220).

CsA treatment for clinical HCV is not ideal because the CsA-CyPA complex also targets calcineurin (123), rendering the treatment inappropriate for immunosuppressed patients. Debio-025 (Alisporivir®, Novartis) is a synthetic, non-immunosuppressive CsA analogue that binds directly to CyPA without binding to calcineurin (100, 110). It is approximately 10-fold more effective against HCV replication in vitro than CsA (36, 153) and effectively reduces HCV RNA in combination with either IFN-α2a in naïve patients (56) or with DAAs in replicon cells, with a very high resistance threshold (36). Like CsA treatment, Debio-025 treatment inhibits HCV replication by competing with NS5A and/or NS5B for binding to CypA (35), and the same resistance mutations occur in NS5A as seen with CsA treatment (30, 35, 100). This is in sharp contrast to resistance to TIZ in that no virus-specific mutations conferring TIZ resistance were identified in HCV isolates from two independent resistant replicon cell lines, NTZ-11 and TIZ-9 (104, 226).

In summary, we have established an anti-HCV mechanism for TIZ, induction of increased NS5A p58 levels. The drug-induced change in p58 is possibly regulated by an indirect effect on the cellular kinase CKIα, which is known to be a primary regulator of NS5A hyperphosphorylation and, therefore, HCV replication. However, due to the lack of a demonstration of a direct effect by the drug on CKIα, the primary cellular target for TIZ remains unknown. Depending on the identity of the primary target and the potential for multiple downstream consequences on various interrelated cellular pathways, it is
likely that different antiviral mechanisms will be associated with the various viruses that TIZ is known to inhibit, related to variations in virus-specific intracellular environments. However, it is our belief that the primary host target (or target family) will be the same for all TIZ-sensitive viruses.
This dissertation offers a contribution to the current understanding of HCV replication. It is well accepted that NS5A p58 inhibits HCV RNA replication by disrupting the replicase complex, and the literature shows a general, whole-cell inhibition of viral RNA replication when p58 levels are increased. Data presented in this dissertation provide a direct link between increased NS5A p58 and decreased synthesis of nascent RNA templates within the replication complex, which, while implied by the established model, was not previously demonstrated.

Much of the seminal work on HCV has employed specific protein expression plasmids, making purification and analysis of viral life cycle components more efficient. However, all of the virus-specific data presented in this dissertation were generated using the Con1 subgenomic replicon, demonstrating the feasibility of conducting research with this model. While notably less efficient and technically more difficult than standardized expression plasmids due to much lower viral protein levels, it is our opinion that it is a more relevant model of HCV replication in cell culture since observations are made in the context of a viral RNA replication cycle. Conclusions drawn from studies using the subgenomic replicons are more readily translatable for clinical relevance.

Screening for new HCV antivirals is typically performed by first analyzing the effects of treatment on viral RNA isolated from cells. The more promising compounds are later analyzed with the in vitro NS3 helicase, NS3 protease, and NS5B polymerase assays. This dissertation demonstrates the utility of an additional in vitro assay for RdRP
activity for antiviral analysis, performed within the context of the viral replication complex, which is more relevant than the traditional \textit{in vitro} RdRP polymerase assay.

To our knowledge, this is the first time a clinically relevant anti-HCV compound has been shown to inhibit HCV replication through modulation of NS5A hyperphosphorylation. These data also provide additional evidence of the feasibility of targeting a host protein critical for HCV replication rather than targeting a viral protein directly. TIZ and Debio-025 are the only compounds with a host target that have been successful in clinical trials. Both TIZ and Debio-025 have very high barriers to viral resistance, both work extremely well in combination with IFN\(\alpha\) and DAAs, and both have low EC\(_{90}\) concentrations. More importantly, both compounds are effective for patients that have previously failed, or are ineligible for, standard treatment.
REFERENCES


91. **Ikeda, M., M. Yi, K. Li, and S. M. Lemon.** 2002. Selectable subgenomic and genome-length dicistronic RNAs derived from an infectious molecular clone of


116. **Liang, S.-H., W. Zhang, B. C. McGrath, P. Zhang, and D. R. Cavener.** 2006. PERK (eIF2 alpha kinase) is required to activate the stress-activated MAPKs and induce the expression of immediate-early genes upon disruption of ER calcium homeostasis. Biochemistry Journal **393**:201-209.


184. **Shimakami, T., M. Hijikata, H. Luo, Y. Y. Ma, S. Kaneko, K. Shimotohno, and S. Murakami.** 2004. Effect of interaction between hepatitis C virus NS5A


