Protease inhibitors block multiple functions of the NS3/4A protease-helicase during the hepatitis C virus life cycle

David R. McGivern¹,², #, Takahiro Masaki¹,², †, William Lovell¹,², Chris Hamlett³, Susanne Saalau-Bethell³ and Brent Graham³

¹Division of Infectious Diseases and ²Lineberger Comprehensive Cancer Center, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA; ³Astex Pharmaceuticals, Cambridge, CB4 0QA, UK.

† Present address: Dept. of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

Running title: Protease inhibitors directly block HCV RNA synthesis

#To whom correspondence should be addressed:

David R. McGivern, Ph.D.

8.001A Burnett-Womack CB #7292

The University of North Carolina at Chapel Hill

Chapel Hill, NC 27599-7292 USA

Tel: 919-843-9958; Fax: 919-843-7240

e-mail: mcgivern@med.unc.edu

Keywords: virus assembly, RNA synthesis, helicase, antiviral, allosteric site

Word count: 3893, Abstract: 250 (Importance: 120)
ABSTRACT

The hepatitis C virus (HCV) NS3 is a multifunctional protein composed of a protease domain and helicase domain linked by a flexible linker. Protease activity is required to generate viral non-structural (NS) proteins involved in RNA replication. Helicase activity is required for RNA replication and genetic evidence implicates the helicase domain in virus assembly. Binding of protease inhibitors (PIs) to the protease active site blocks NS3-dependent polyprotein processing but might impact other steps of the virus life cycle.

Kinetic analyses of antiviral suppression of the cell culture-infectious gt1a strain H77S.3 were performed using assays that measure different readouts of the viral life cycle. In addition to the active site PI telaprevir, we examined an allosteric protease/helicase inhibitor (APHI) that binds a site in the interdomain interface.

By measuring nucleotide incorporation into HCV genomes, we found that telaprevir inhibits RNA synthesis as early as 12 hrs, at high but clinically relevant concentrations. Immunoblot analyses showed that NS5B abundance was not reduced until after 12 hrs suggesting that telaprevir exerts a direct effect on RNA synthesis. In contrast, the APHI could partially inhibit RNA synthesis suggesting that the allosteric site is not always available during RNA synthesis. The APHI and active site PI were both able to block virus assembly soon (<12 hrs) after drug treatment suggesting that they rapidly engage with and block a pool of NS3 involved in assembly. In conclusion, PIs and APHIs can block NS3 functions in RNA synthesis and virus assembly in addition to inhibiting polyprotein processing.
The NS3/4A protease of hepatitis C virus (HCV) is an important antiviral target. Currently, three PIs have been approved for therapy of chronic hepatitis C and several others are in development. NS3-dependent cleavage of the HCV polyprotein is required to generate the mature non-structural proteins that form the viral replicase. Inhibition of protease activity can block RNA replication by preventing expression of mature replicase components. Like many viral proteins, NS3 is multifunctional but how PIs affect stages of the HCV life cycle beyond polyprotein processing has not been well studied. Using cell-based assays, we show here that PIs can directly inhibit viral RNA synthesis and also block a late stage in virus assembly/maturation at clinically relevant concentrations.
INTRODUCTION

Chronic infection with the hepatitis C virus (HCV) is a leading cause of end stage liver disease and hepatocellular carcinoma. HCV is an RNA virus with a cytoplasmic life cycle and therapies that prevent virus replication can ultimately eradicate the virus from the host, reducing both the development of liver disease and the risk of cancer. The former standard of care for chronic hepatitis C was dual therapy with pegylated interferon α and ribavirin but this was lengthy, poorly tolerated, and only effective in <50% of persons infected with the most common HCV genotypes. Over the past decade, intensive research efforts directed at understanding the HCV life cycle have resulted in the development of small molecule inhibitors targeting specific viral proteins including the NS3 protease and the NS5B RNA-dependent RNA polymerase (1).

Some of these direct-acting antiviral (DAA) drugs have already been approved for use in therapy and several other DAAs are in clinical development.

The non-structural- (NS-)3 protein has emerged as a key target for antiviral drug development. The genome of HCV encodes a single polyprotein that is co- and post-translationally cleaved into 10 individual proteins by cellular and viral proteases. The HCV NS3 protein, together with its cofactor NS4A is a serine protease that is required to cleave the polyprotein at four sites in order to generate viral proteins essential for replication of the RNA genome. In addition, NS3 cleaves the adaptor proteins MAVS (2) and TRIF (3) to block activation of interferon gene expression through the retinoic acid inducible-I (RIG-I) and Toll-like Receptor 3 (TLR3) pathways. Thus, the NS3 protease is a particularly attractive target for antiviral intervention since its inhibition not only interferes with polyprotein processing but also restores antiviral signaling (4, 5). The first direct-acting antiviral drugs to be approved for the therapy of chronic hepatitis C, boceprevir (6) and telaprevir (7), are both peptidomimetic linear
ketoamides that target the active site of the protease domain of NS3. Further development of protease inhibitors (PI) with macrocycles at either P1-P3 or P2-P4 resulted in improved antiviral potency. Recently, simeprevir (8) became the first macrocyclic PI to be approved for treatment of chronic hepatitis C in the USA (9). Several other PIs are in clinical development including the next generation PI grazoprevir (10).

Although the protease activity of NS3 has been the focus of drug development efforts, NS3 is a bifunctional enzyme with separate protease and helicase domains connected by a flexible linker. The helicase domain has NTPase and 3’-5’ RNA unwinding activity (11). The ATP-dependent RNA unwinding activity of the NS3 helicase is essential for HCV RNA synthesis (12), and genetic and biochemical studies have implicated the NS3 helicase domain in viral assembly, independent of its role in RNA synthesis (13).

The two domains can be separated and their enzymatic activities studied in vitro but their attachment in the full-length NS3 has been shown to strongly influence their individual properties. For example, the isolated helicase domain preferentially unwinds DNA substrates but the presence of the protease domain in full-length NS3 can alter substrate selectivity and enhance RNA binding and unwinding (14). Conversely, the helicase domain has been shown to stimulate protease activity in the context of full length protein (15), and polynucleotides, especially polyuracil, have been shown to stimulate protease activity of the full-length NS3 but not the activity of the isolated protease (16). A recent mutational analysis has shown that the linker region connecting the two domains is not required for protease or helicase activity but is critical for replication and infectivity (17). These data support a role for the linker either in modulating conformation of full length NS3 or in mediating interactions between NS3 and other viral or host proteins during the HCV life cycle.
Recently, a novel class of allosteric protease/helicase inhibitors was identified using fragment-based screening and structure-guided design (18). These inhibitors bind at the interface between the protease and helicase domains of NS3, a region proposed to modulate the activities of the two enzyme domains in vivo (19).

To characterize the mechanism of action of allosteric NS3 inhibitors, a representative compound from this class, AT23708, was compared to the active site PI telaprevir in multiple assays of antiviral activity that specifically examine different steps in the viral life cycle, including polyprotein synthesis, RNA synthesis, and the intracellular assembly and release of infectious genotype 1a virus H77S.3. These assays not only allowed characterization of the allosteric NS3 inhibitor but also revealed unexpected antiviral activities of the PI telaprevir on NS3-dependent stages of the viral life cycle in addition to polyprotein processing.

Both classes of inhibitor were able to inhibit a late stage in virus assembly or maturation within 12 hrs following addition of drug to infected cells. Additionally, RNA synthesis was almost completely blocked by telaprevir and partially blocked by AT23708 within 12 hrs – a time point when there is little detectable reduction in intracellular NS5B levels. These data show that protease inhibitors targeting the multifunctional NS3 protein have an early effect on RNA synthesis in addition to polyprotein processing.

**MATERIALS AND METHODS**

**Cell Culture and Inhibitors.**

The Huh7 sub-clone, 2-3c (20) is a cured replicon cell line that is highly permissive for HCV replication and shows very weak RIG-I signaling following challenge with Sendai virus (21). 2-3c cells were grown in Dulbecco’s Modified Eagle’s Medium (Gibco-BRL)
supplemented with 10% fetal calf serum, 100U penicillin, 100U streptomycin, 1mM L-glutamine, 1X non-essential amino acids, 1µM vitamin E, 1mM sodium pyruvate and 10 mM HEPES buffer pH 7.4. For virus production assays, concentration of HEPES buffer pH 7.4 was increased to 50mM. AT23708, an allosteric NS3 inhibitor (18) and the active site PI telaprevir (7) were synthesized at Astex Pharmaceuticals (Cambridge, UK). The non-nucleoside polymerase inhibitor HCV796 (22) was a gift from Anita Howe (Merck Research Laboratories, Kenilworth, NJ). Stock solutions of telaprevir, AT23708 and HCV796 were prepared in DMSO.

Virus infections and antiviral assays.

H77S.3 is a cell-culture adapted infectious molecular clone of a genotype 1a HCV (23, 24). H77S.3/GLuc2A is a modified H77S.3 that expresses Gaussia luciferase (GLuc) as a fusion with its polyprotein. In this genome, the GLuc sequence, followed by a foot and mouth disease virus 2A autoprotease, is inserted in frame between the p7 and NS2 sequences of H77S.3. Cleavage events required for production of mature secreted GLuc are independent of HCV protease activity. Plasmids encoding H77S.3 or H77S.3/GLuc2A genomes were linearized by XbaI digestion and transcribed in vitro using the T7 Megascript kit (Ambion). RNA products were DNase treated and purified using the Rnaseasy Mini kit (Qiagen), and electroporated into Huh7 cells as described previously (25). Electroporated cells were cultured for 7 days to allow HCV replication to reach a steady state before seeding to different plate formats for specific assays of the virus life cycle. GLuc assays of HCV genome replication and measurement of infectious HCV production by focus-forming unit (FFU) assay, have been described previously (26). To measure effects of protease inhibitors on cell proliferation and viability, a WST-1 assay was used according to the manufacturer’s protocol (Roche).
Western blot analyses of viral protein abundance.

Cells were washed twice in 1x PBS and directly lysed in 1x Laemmlli sample buffer containing 5% β-mercaptoethanol and passed through a QIAshredder (Qiagen) to reduce sample viscosity. Samples corresponding to one tenth of a 12-well plate were resolved by SDS-PAGE on a 4-15% Tris-glycine gradient gel (BioRad) and total protein transferred to low fluorescence PVDF membrane using the Transblot Turbo Transfer System (BioRad) according to manufacturer’s instructions. Blots were probed with primary antibodies against HCV NS5B (rabbit polyclonal, ab65410; Abcam) and β-actin (mouse monoclonal, AC-15; Sigma Aldrich). Secondary antibodies were IRDye 800CW goat anti-mouse IgG and IRDye 680 goat anti-rabbit IgG. Blots were visualized by two color detection using an Odyssey Infrared Imaging System and bands quantified using Odyssey v3.0 software (LI-COR, Inc.). For each dose of inhibitor, NS5B abundance was normalized to β-actin abundance in the same lane as a loading control.

Statistical analyses.

To determine the 50% (EC₅₀) and 90% (EC₉₀) effective concentrations of telaprevir and AT23708 in the different assays (GLuc, immunoblot quantitation of residual NS5B abundance, FFU, RNA synthesis), data were fit to a four-parameter dose response curve with variable slope using Prism 6.0 for Windows (GraphPad Software, Inc). Values reported are the estimated concentration ± 95% confidence interval. Maximum achievable inhibitory activities (Eₘₐₓ values) at different time points were compared by Mann-Whitney test using Prism 6.0.

Measurement of RNA synthesis inhibition.
To determine the impact of NS3 inhibitors on viral RNA synthesis, nascent RNA was labeled by incubating HCV-infected cells with 5-ethynyl uridine (EU) from 2-12 hrs after addition of inhibitor. Total RNA was purified from cells using the RNeasy Mini Kit (Qiagen). EU-labeled RNA was conjugated to biotin and isolated from the total RNA using the Click-iT Nascent RNA Capture Kit (Invitrogen). Newly synthesized, EU-labeled HCV RNA and total cell-associated HCV RNA were quantified using HCV-specific primers and a 2-step quantitative RT-PCR assay described previously (23).

Rate-zonal gradient analyses.

Cells were transfected with H77S.3 as described above and treated for 12 hrs with either 0.1% DMSO or NS3 inhibitors at concentrations that represent 5x the EC90 of those drugs as measured by GLuc assay. After drug treatment, cells were harvested by trypsinization, washed twice in PBS and resuspended in 400µl PBS. Lysates were prepared by multiple freeze thaw cycles as described previously and subjected to rate zonal centrifugation. Clarified lysate (350µl) was loaded onto a 10-50% sucrose gradient prepared in TNE (10 mM Tris, pH 8.0, 150 mM NaCl, 2 mM EDTA) and centrifuged for 1 hour at 40,000 rpm in a SW55 Ti rotor at 4°C. For each gradient, 20 fractions collected from the top of the gradients and analyzed for HCV RNA by qRT-PCR (23) and for infectivity by FFU assay.

RESULTS

Kinetics of antiviral suppression by different classes of inhibitor that target NS3.
As a first step to characterize how inhibitors that bind at different sites on the NS3 protein impact different aspects of the HCV life cycle, kinetics of antiviral activity were monitored by measuring secreted *Gaussia* luciferase (GLuc) activity from cells infected with the gt 1a cell culture infectious H77S.3/GLuc2A at different time points following treatment with either telaprevir (a linear ketoamide that binds the active site of the NS3 protease domain) or AT23708 (an APHI that binds NS3 at the interface of the protease and helicase domains). H77S.3/GLuc2A is a modified version of H77S.3 in which the GLuc coding sequence has been inserted between p7 and NS2, followed by the foot-and-mouth disease virus 2A protein coding sequence. When the H77S.3/GLuc2A RNA is transfected into Huh7 cells, the RNA can replicate and GLuc translated from the replicating genomes is secreted into the medium. Secreted GLuc is a measure of polyprotein synthesis but this correlates well with intracellular RNA levels. Thus, HCV RNA abundance and polyprotein synthesis can be monitored by measuring GLuc activity in media collected at 12 or 24 hour intervals following transfection.

In this assay, telaprevir and AT23708 displayed different kinetics of antiviral suppression (Figure 1A and Table 1). This difference in kinetics can be seen most easily by comparing maximum achievable inhibition (E\text{max}) values for both drugs in the GLuc assay at different time points (Table 4). E\text{max} values were significantly higher for telaprevir compared to AT23708 at both 24 hrs (81% vs 38%; p=0.0022) and 48 hrs (99% vs 90%; p=0.0022).

WST-1 assays were performed in parallel to determine the impact of telaprevir and AT23708 on cell viability and proliferation (Figure 1C). No change in cellular proliferation was observed at 12 or 24 hrs for either inhibitor. At 48 and 72 hrs, a modest 20-30% reduction in cell viability/proliferation was observed only at the highest concentrations tested (25 or 50 µM) for
both inhibitors. The 50% cytotoxic concentrations (CC$_{50}$) for both inhibitors were greater than 50 µM, the highest concentration tested.

Reduction of intracellular non-structural protein levels.

Active site protease inhibitors such as telaprevir inhibit the NS3-dependent cleavage events that generate mature NS proteins required for RNA genome replication. AT23708 also inhibits NS3-dependent proteolytic processing *in vitro* (18). Western blot analyses were used to compare the rates at which each inhibitor reduces intracellular non-structural protein levels.

Huh7 cells were electroporated with H77S.3 RNA genomes and cultured for one week to allow virus replication to stabilize. Infected cells were mock-treated (0.1% DMSO) or treated with telaprevir or AT23708 at concentrations ranging from 25 nM to 50 µM for 72, 48, 24 or 12 hrs prior to harvest, at which time, cell lysates were prepared and subjected to SDS-PAGE and Western blotting. Membranes were probed for HCV NS5B or core protein with actin or β-tubulin as loading controls.

Reductions of NS5B protein abundance by both telaprevir and AT23708 were slow (Figure 1B and Table 2). In agreement with GLuc assays (Figure 1A), reduction of NS5B abundance was slightly faster following addition of telaprevir compared to AT23708. As was the case for the GLuc assay, the difference in kinetics can be seen most easily by comparing $E_{\text{max}}$ values for both drugs in the NS5B Western blot assay at different time points (Table 4). $E_{\text{max}}$ values were significantly higher for telaprevir compared to AT23708 at both 24 hrs (85% vs 48%; p=0.0022) and 48 hrs (100% vs 78%; p=0.0159). Importantly, at 12 hrs after addition of either drug there was little detectable reduction in intracellular NS5B abundance. This observation allowed further analyses to be performed focusing on RNA synthesis and infectious
virus production at early time points after addition of drug when protease inhibition is not yet resulting in significant decreases in NS5B abundance.

**Inhibition of RNA synthesis by DAAs targeting NS3.**

To directly investigate inhibition of RNA synthesis, nascent RNA was labeled by incubating HCV-infected cells with 5-ethynyl uridine (EU) from 2 to 12 hrs after addition of inhibitor. Total RNA was extracted from the cells and following isolation of EU-labeled RNA, newly synthesized HCV RNA genomes were quantified by qRT-PCR. Total residual HCV RNA abundance was also quantified by qRT-PCR at 12 hrs after inhibitor addition (Figure 2A). Reduction of newly synthesized HCV RNA (Figure 2B) was faster than reduction of total residual HCV RNA for both inhibitors suggesting that they primarily act to reduce new RNA synthesis and do not accelerate degradation of existing viral RNA genomes. Telaprevir was able to almost completely inhibit HCV RNA synthesis (Emax 92%) within 12 hrs of drug addition at high but clinically relevant concentrations (Figure 2B, left hand panel). These data suggest that telaprevir can directly interfere with NS3 activity during RNA synthesis independently of its inhibitory activity on proteolytic processing since at 12 hrs after addition of drug there is little reduction of intracellular NS5B (Figure 1B).

In contrast to telaprevir, AT23708 showed only partial inhibition of HCV RNA synthesis at 12 hrs after addition (Emax 53%; Figure 2B, right hand panel). The difference between telaprevir and AT23708 in early kinetics of RNA synthesis inhibition correlates with the difference in kinetics observed in the GLuc assay and is consistent with a model in which binding of AT23708 to NS3 is conformation-dependent (see Discussion).
Inhibition of virus production by DAAs targeting NS3.

To compare inhibition of virus production by the active site PI and allosteric NS3 inhibitor, an FFU assay was used to measure infectious virus produced from H77S.3 infected cells at different 12-24 hr intervals following addition of inhibitors. Inhibition of virus production was rapid and occurred sooner than inhibition of RNA genome replication for both telaprevir and AT23708 (Figure 3A and Table 3). The difference between the timing of inhibition of RNA synthesis and virus production was particularly striking for AT23708.

Complete inhibition of virus production was observed with AT23708 by 12hrs after addition, a time point where there was incomplete inhibition of RNA synthesis (Figure 2) and no reduction in intracellular non-structural protein levels (Figure 1B). Telaprevir was also able to block virus production at 12 hrs after addition (Figure 3) but at this time point, telaprevir can also inhibit RNA synthesis (Figure 2B). Several further lines of evidence suggest that telaprevir has an additional effect on virus production. First, the EC$_{50}$ at 12 hrs in the virus production (FFU) assay is 777 nM (Table 3) compared to 2400 nM in the RNA synthesis assay (Figure 2). Furthermore, at very early time points following addition of inhibitor at 5xEC90, both telaprevir and AT23708 were able to block virus production faster than the RNA-dependent RNA polymerase inhibitor HCV796 (Figure 3B). These data suggest that telaprevir is also able to inhibit virus production in addition to blocking RNA synthesis and polyprotein processing.

Comparing different assays, E$_{\text{max}}$ values (Table 4) were significantly greater when measured in the virus production assay compared to the GLuc or NS5B Western blot assays for telaprevir at 12 and 24 hrs and for AT23708 at 12, 24 and 48 hrs (p<0.01 in every case).

A potential caveat in this experiment is that infectious virus production is measured in medium containing inhibitor. In the virus production assay, H77S.3-infected cells were treated...
with different concentrations of protease inhibitor and medium was harvested at various times after treatment. Virus released into the medium was used to inoculate naïve cells for 4 hrs before washing off the inoculum, replacing with fresh medium. After a further 3 days of culture, inoculated cells were stained for core protein to quantify infectious foci. Although the inoculum was washed out in this experimental design, it is possible that residual amounts of inhibitor were being carried over during the 4 hr inoculation period and blocking cleavage of polyprotein translated from newly uncoated viral RNA following entry. To determine the contribution of drug carry-over in this assay, virus inoculum produced from cells grown in the absence of drug was spiked with an equal volume of conditioned cell culture medium from mock-infected cells treated for 12 hrs with a range of inhibitor concentrations and this mixture was used to inoculate naïve cells for 4 hrs and virus infectivity assessed by FFU assay (Figure 3A, “carry-over”).

Inhibition of virus infectivity by drug carry-over was only observed at concentrations of 12.5 µM or higher for both telaprevir and AT23708: approximately 20- or 100-fold higher than the concentrations required to block virus production. These data confirm that the rapid decrease in FFU following protease inhibitor treatment is a genuine effect on virus production and not simply due to inhibition of virus infectivity caused by drug carry-over.

During the assembly of HCV, NS3 is recruited to lipid droplets along with the NS5A protein (27). Our previous studies suggested that production of HCV particles is a dynamic process that can be rapidly inhibited by DAAs that target viral proteins involved in assembly such as NS5A (26). Whether inhibitors that target NS3 impact viral assembly or maturation is not known but genetic and biochemical studies have suggested a role for the helicase domain of NS3 in virus assembly (13). Thus, it is possible that inhibitors targeting NS3 could have a direct effect on intracellular assembly. To assess this, infected cells were treated with a high
concentration (5 µM; > 5x EC90 in GLuc assay at 72 hrs) of telaprevir or AT23708 for 12 hrs.

The cells were then lysed by repeated freeze-thaw cycles in the absence of detergent, and intracellular viral particles separated by rate-zonal ultracentrifugation through a sucrose gradient. Fractions collected from the top of the gradient were analyzed by qRT-PCR to determine HCV genomic RNA content and FFU assay to determine infectious virus content (Figure 4). Although the total amount of viral RNA was reduced by 40-45% in lysates from inhibitor-treated cells versus cells treated only with DMSO, the presence of discrete peaks of HCV RNA in fractions 7 and 10 of gradients suggested continued assembly of RNA-containing particles in the presence of inhibitor. Infectious virus was clearly present and sedimenting with the second RNA peak in fractions 9-10 of the gradient loaded with lysate from mock-treated cells. Importantly, however, there was a sharp reduction of infectious virus in the fractions from cells treated with AT23708 or telaprevir.

Collectively, the data suggest that NS3 inhibitors block a late step in the intracellular assembly and/or maturation of virus, resulting in early and nearly complete inhibition of production of infectious virus.

**DISCUSSION**

In this study, the active site PI telaprevir was compared with a novel allosteric NS3 inhibitor in cell based assays that focus on different aspects of the HCV life cycle. Many viral proteins are multifunctional and DAAs may hit one or multiple functions of their target protein. The use of multiple assays to probe the HCV life cycle in detail can help understand how DAAs interfere with the life cycle and illuminate the role of specific proteins in virus replication.
Protease inhibitors can directly interfere with RNA synthesis

In addition to blocking the NS3-dependent cleavage of the HCV polyprotein, the active site PI telaprevir was found to inhibit new RNA synthesis and virus production prior to egress at a late stage during assembly or particle maturation. Since the HCV RNA-dependent RNA polymerase NS5B is generated by NS3-dependent cleavage of the polyprotein, inhibition of RNA synthesis by a PI such as telaprevir could be a consequence of depletion of intracellular NS5B. However, inhibition of RNA synthesis by telaprevir occurred at 2-12 hrs after addition of the drug, a time point when there is little reduction in NS5B abundance, suggesting that telaprevir can directly inhibit RNA synthesis. This activity of an active site PI is unexpected but is consistent with a role for protease domain during RNA synthesis. Previous biochemical studies have shown that the protease domain of NS3 is required for efficient unwinding of double-stranded (ds) RNA by the helicase (28) and recently, Aydin et al. (19) proposed a model in which the protease domain of NS3 binds to dsRNA as a clamp while the helicase unwinds the ds template to allow access by NS5B to the negative strand for RNA synthesis. It is tempting to speculate that binding of telaprevir to the protease domain may inhibit RNA synthesis by reducing helicase activity but a previous study has reported that telaprevir does not inhibit the helicase activity of full length NS3 in vitro (29). In vivo, however, NS3 functions as part of a multi-protein replicase complex where binding of telaprevir may result in disruption of protein-protein and protein-RNA interactions to block a function of NS3 in RNA synthesis. An alternative explanation is that the majority of new RNA synthesis is performed by newly synthesized NS5B. Further experiments will be required to test these hypotheses.

The APHI AT23708, already known to act as an inhibitor of polyprotein processing, was also able to inhibit RNA synthesis at early time points following addition of drug but unlike
telaprevir, the inhibition was only partial. This difference may be explained by the conformation-dependent binding of the APHI to NS3 which targets a site in the interdomain interface that is present in the compact crystallographic conformation of NS3 but not in the extended conformation, which biochemical studies suggest represents the active conformation during RNA synthesis (30). Partial inhibition of RNA synthesis by AT23708 is consistent with NS3 adopting the extended conformation at least some of the time during RNA synthesis.

Protease inhibitors can interfere with virus assembly/maturation

Both telaprevir and AT23708 were able to rapidly inhibit virus production from infected cells. Inhibition occurred at early time points (12 hrs after drug addition) when there was little reduction of residual HCV RNA in the cells and no reduction in intracellular levels of non-structural proteins. Analyses of very early time points showed onset of inhibition by 3 hrs following addition of drug and 90% inhibition by 9 hrs. The different kinetics of virus assembly inhibition compared to inhibition of RNA synthesis and reduction of NS5B abundance suggest that (i) assembly is a highly dynamic process and particles are exported rapidly from the cell and (ii) the pool of NS3 involved in virus assembly is distinct from the pool of NS3 involved in RNA synthesis and polyprotein processing.

Our data show that inhibitors targeting NS3 at either the active site or an allosteric site in the interdomain interface can block virus production at a late step during assembly/maturation of the virus particles. While this specific effect of NS3/4A inhibitors has not been demonstrated previously, it is not surprising as NS3 is recruited to lipid droplets together with NS5A and appears to function directly in viral assembly (13, 27). Some variants associated with resistance to NS3/4A inhibitors also impair viral fitness by negatively impacting steps involved in the release of infectious virus (23). Our data are in agreement with a previous study (31) that focused
on daclatasvir but also included data suggesting telaprevir can block virus production using 391 mathematical modeling analyses of rates of viral RNA decline in patient serum following 392 telaprevir treatment. Those modeling analyses predicted that telaprevir can inhibit virus 393 assembly/secretion more effectively than polymerase inhibitors but that this effect was weaker 394 than for NS5A inhibitors such as daclatasvir.

How might inhibitors targeting NS3 block assembly? It is possible that binding of 396 telaprevir to the active site or AT23708 to the allosteric site inhibits virus release by interfering 397 with an activity of the protease in viral assembly/release. NS3 is a multifunctional protein and 398 the activity of its helicase is regulated through its protease-helicase domain interface (19). The 399 binding of a small molecule to the protease domain or at the interdomain interface might thus 400 alter helicase activity during these final steps in the viral life cycle. The interaction of inhibitors 401 with NS3 may prevent an interaction of the helicase domain with core protein, which genetic 402 (32) and biochemical (33) studies suggest is required for particle assembly. The effect of 403 telaprevir and AT23708 on viral release could also occur indirectly via NS5A, as NS5A 404 hyperphosphorylation is dependent upon NS3 protease activity (34) and its absence might affect 405 NS5A functions involved in viral assembly and release. Further experiments will be required to 406 distinguish between these possibilities.

The protease activity of NS3/4A is responsible for blunting RIG-I dependent host innate 408 immune responses through cleavage of the signaling adaptor molecules MAVS (2). Two 409 previous studies have demonstrated that small molecule inhibition of NS3 can restore host cell 410 innate immune signaling by preventing cleavage of MAVS (4, 5). Kinetic analyses demonstrated 411 that restoration of signaling was faster with a protease inhibitor (2-4 days depending upon 412 concentration) compared to a polymerase inhibitor (7 d; (4)). Importantly, the rapid effects (<12
hrs) on virus production and RNA synthesis observed in the present study precede (and are thus likely to be independent from) restoration of innate immune signaling observed in previous studies. PI-mediated restoration of MAVS signaling may be slower than direct PI effects on the viral life cycle because it requires several events including synthesis of new MAVS protein, trafficking of MAVS and accumulation at the mitochondria-associated membranes (35), interaction with RIG-I molecules engaged with the HCV PAMP and subsequent downstream signaling to promote expression of antiviral genes (36).

The HCV genome encodes only ten mature proteins and many of these proteins have to be multi-functional to perform all of the activities required to complete the viral life cycle. For example, NS5A is involved in assembly of new replicase complexes but also required for virion assembly and inhibitors targeting NS5A can block both of these processes (26, 31, 37). NS3 has been termed the “Swiss Army knife” of HCV because of its multiple roles in the viral life cycle (38). The current study has provided novel insight into how NS3 inhibitors impact specific steps in the viral life cycle and demonstrates that their mechanism of action is more complex than simply blocking NS3-dependent polyprotein cleavage to prevent replicase complex formation.

Acknowledgements

The authors thank Drs. Zongdi Feng and Asuka Yuki-Hirai for expert advice on rate zonal centrifugation analyses and Dr. Stanley Lemon for helpful discussions. DRM was funded by a research grant from Astex Pharmaceuticals and the University Cancer Research Fund of UNC Chapel Hill.

REFERENCES


Figure Legends

Figure 1: (A) Kinetic analysis of the suppression of H77S.3/GLuc2A virus replication by the active site protease inhibitor telaprevir or the allosteric NS3 inhibitor AT23708. Huh7 cells were electroporated with H77S.3/GLuc2A RNA genomes and passaged for one week before challenging with a range of inhibitor concentrations. At 12, 24, 48 and 72 hours following addition of inhibitor, cell culture media were harvested and replaced with fresh medium containing inhibitor. Percent inhibition of GLuc activity in cell culture media is plotted on the y-axis, where 0% is GLuc activity in cells treated with medium containing DMSO vehicle only (no inhibitor) and 100% inhibition is a reduction of GLuc activity to background levels (determined by measurement of GLuc activity in medium from cells transfected with a replication-incompetent RNA genome, H77S-AAG/GLuc2A). Data shown represents mean ± s.e.m. from 3 replicates in a representative experiment from multiple experiments. Data were fit to dose response curves as described in the Materials and Methods. EC<sub>50</sub> and E<sub>C90</sub> values for telaprevir and AT23708 in the GLuc assay are shown in Table 1. (B) Reduction of intracellular NS5B abundance following addition of telaprevir or AT23708 to Huh7 cells infected with H77S.3. NS5B and β-actin were detected in cell lysates by Western blot. NS5B levels were normalized using β-actin as a loading control. Normalized NS5B abundance was expressed as percent.
inhibition where 0% is NS5B abundance in cells treated with DMSO vehicle only and 100% inhibition is reduction of NS5B levels to background levels (determined using lysates from uninfected cells). Data shown represents mean ± s.e.m. percent inhibition from 3 independent experiments. EC50 and EC90 values for telaprevir and AT23708 in the Western blot analyses are shown in Table 2. (C) Effect of telaprevir and AT23708 on cell proliferation and viability measured by WST-1 cell proliferation assay. Data shown are normalized to values obtained from cells grown in medium containing 0.1% DMSO vehicle and represent mean ± s.e.m. from 3 independent experiments.

Figure 2: (A) Reduction of total residual HCV RNA in H77S.3-infected Huh7 cells by telaprevir or AT23708 at 12 hrs (B) Inhibition of new HCV RNA synthesis in H77S.3-infected Huh7 cells by telaprevir or AT23708 between 2 and 12 hrs. Shaded area in the left hand panel represents previously reported range of telaprevir concentrations (Cmin 3.0 µM, Cmax 5.2 µM) in plasma of persons treated with 750 mg telaprevir every 8h (4). Data shown in (A) and (B) represent mean ± s.e.m. percent inhibition from 3 experiments and were fit to a dose-response curve as in Fig. 1.

Figure 3: Rapid inhibition of virus production by telaprevir and AT23708. (A) Reductions in infectious virus released into supernatant fluids between 0-12, 12-24, 24-48, and 48-72 hrs after challenging infected cell cultures with a range of concentrations of telaprevir or AT23708. Infectious virus release was quantified by FFU assay. Reduction in virus infectivity caused by residual inhibitor present in the inoculum was measured and plotted as “carry-over”. Results are shown as percent inhibition where 0% is FFU/ml released from infected cells grown in the absence of inhibitor (DMSO vehicle only) and 100% inhibition is a reduction of virus production.
to below limits of detection (<10 FFU/ml). Data shown represent mean ± s.e.m. of 2-3 independent results and were fit to a dose-response curve as in Fig. 1. EC_{50} and EC_{90} values for telaprevir and AT23708 in the FFU assay are shown in Table 3. (B) Very early inhibition of virus production by telaprevir and AT23708 compared to the non-nucleoside RdRP inhibitor HCV796. Inhibitors were added at 2 hr intervals to H77S.3-infected cells at concentrations equivalent to 5x EC_{90} in the 72 hr GLuc assay. At 6 hrs, medium was removed from all cultures and replaced with fresh medium containing the inhibitors. Supernatant fluids were harvested 3 hrs later and infectious virus quantified by FFU assay.

**Figure 4:** Rate-zonal centrifugation of cell lysates derived from H77S.3-transfected cells that were either mock-treated (A) or treated with 5 µM telaprevir (B) or 5 µM AT23708 (C) for 12 hrs. Fractions collected from the top of the gradients were tested for HCV RNA by qRT-PCR and for infectivity by FFU assay.
<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>EC₅₀ (nM)</th>
<th>EC₉₀ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 hrs</td>
<td>24 hrs</td>
</tr>
<tr>
<td>Telaprevir</td>
<td>&gt;50,000 †</td>
<td>2180</td>
</tr>
<tr>
<td>AT23708</td>
<td>&gt;50,000 †</td>
<td>50000 †</td>
</tr>
</tbody>
</table>

* (95% confidence interval).
† Highest tested concentration.
Table 2. EC$_{50}$ and EC$_{90}$ of NS3 inhibitors against H77S.3 virus (NS5B reduction assessed by Western blot).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>12 hrs (nM)</th>
<th>24 hrs (nM)</th>
<th>48 hrs (nM)</th>
<th>72 hrs (nM)</th>
<th>12 hrs (nM)</th>
<th>24 hrs (nM)</th>
<th>48 hrs (nM)</th>
<th>72 hrs (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telaprevir</td>
<td>25760</td>
<td>1076 (241-4803)</td>
<td>608 (97-3823)</td>
<td>1099 (908-1329)</td>
<td>&gt;50000$^\dagger$</td>
<td>28729 (327-50000)</td>
<td>1.878 (112-50000)</td>
<td>2366 (1455-3846)</td>
</tr>
<tr>
<td>AT23708</td>
<td>&gt;50000$^\dagger$</td>
<td>351 (120-1027)</td>
<td>399 (192-830)</td>
<td>364 (161-822)</td>
<td>&gt;50000$^\dagger$</td>
<td>&gt;50000$^\dagger$</td>
<td>&gt;50000$^\dagger$</td>
<td>1750 (357-8588)</td>
</tr>
</tbody>
</table>

$^\dagger$(95% confidence interval).

$^\dagger$Highest tested concentration.
### Table 3. EC$_{50}$ and EC$_{90}$ of NS3 inhibitors against H77S.3 virus (FFU assay).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>12 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
<th>12 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telaprevir</td>
<td>777 (686-880)</td>
<td>677 (626-871)</td>
<td>765 (695-949)</td>
<td>738 (666-843)</td>
<td>778 (685-962)</td>
<td>1389 (1180-1635)</td>
<td>1255 (1080-1458)</td>
<td>2493 (2085-3701)</td>
</tr>
<tr>
<td>AT23708</td>
<td>218 (200-237)</td>
<td>91 (81-101)</td>
<td>108 (97-121)</td>
<td>123 (98-105)</td>
<td>491 (413-583)</td>
<td>428 (328-558)</td>
<td>364 (285-466)</td>
<td>294 (253-342)</td>
</tr>
</tbody>
</table>

*(95% confidence interval).*
Table 4. Maximum inhibitory activity (E_{max}) observed at different time points in different assays for Telaprevir and AT23708

<table>
<thead>
<tr>
<th></th>
<th>12 hrs</th>
<th>24 hrs</th>
<th>48 hrs</th>
<th>72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Telaprevir</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GLuc assay</td>
<td>16.67 (±3.08)</td>
<td>80.83 (±2.09)</td>
<td>99.01 (±0.28)</td>
<td>99.94 (±0.02)</td>
</tr>
<tr>
<td>NSSB (Western blot)</td>
<td>34.29 (±6.18)</td>
<td>84.99 (±7.61)</td>
<td>100 (±0.01)</td>
<td>100 (±0.27)</td>
</tr>
<tr>
<td>Virus production (FFU)</td>
<td>100 (±0.01)</td>
<td>100 (±0.01)</td>
<td>100 (±0.01)</td>
<td>100 (±0.01)</td>
</tr>
</tbody>
</table>

| **AT23708**     |              |              |              |              |
| GLuc assay      | 19.16 (±3.10)| 38.06 (±3.39)| 89.99 (±2.87)| 99.54 (±0.39)|
| NSSB (Western blot) | 27.92 (±0.97)| 48.39 (±2.24)| 77.64 (±1.45)| 100 (±0.44)  |
| Virus production (FFU) | 99.89 (±0.07)| 100 (±0.01)  | 99.71 (±0.26)| 100 (±0.01)  |

Data shown represent mean E_{max} values (±sem) from ≥3 experiments.