Oral Session 2: Hepatitis Viruses

Chairs: Phillip Furman, Ph.D. and Klaus Klumpp, Ph.D., 2:00–4:00 pm, Grand A

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Diphenylcarboxamides as Inhibitors of HCV Non-Structural Protein NS5a

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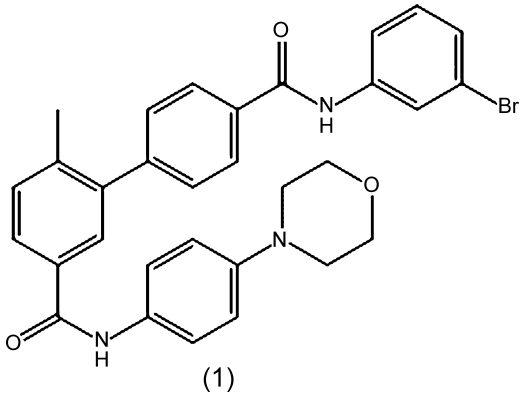
In order to identify novel agents for the treatment of hepatitis C (HCV) a series of analogues were screened in a genotype 1b replicon assay. Rather than directly targeting the protease or polymerase genes, we set about testing families of compounds known to bind to ATP recognition sites (i.e. biphenyl-dicarboxamide derivatives), as it is known that a number of processes vital to viral replication require ATP. The SAR of these molecules will be discussed in detail. Subsequent to the identification of these molecules, their anti-viral mechanism of action was determined by sequencing mutant replicons generated in the presence of compound. Using a simple 2D amide array the first micromolar inhibitor (1) was identified. Subsequent structural modification over a number of iterative cycles gave nanomolar inhibitors. When mutant replicon was generated and then sequenced, the major changes were found to occur in Domain 1 of the NS5a gene. The resistance phenotype was due to Y93C or H mutation, and this mechanism of action has been confirmed by reverse genetics. A compound from this series has been progressed into clinical trials, the results of which will be published in due course.



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Selection of Clinically-relevant Protease Inhibitor Resistant Viruses using the HCV Infection System

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Treatment of HCV patients with direct acting antivirals can lead to the emergence of drug-resistant variants which may pose a long term threat to viral eradication. HCV replicons have been used to select resistance mutations; however, genotype 2 JFH-based viruses provide the opportunity to perform resistance selection in a bona fide HCV infection system. In this study, we used J6/JFH-1 virus to select resistance to the NS3 protease inhibitors BILN-2061 and VX-950. Lunet-CD81 cells were infected with an adapted J6/JFH-1 virus and maintained in the presence of inhibitors until high-titer viral supernatant was produced. Viral supernatants were passaged over naïve cells at escalating drug concentrations and the resulting viruses were then characterized. Phenotypic analyses indicated the selected viruses had comparable infection kinetics to the parental virus but were significantly less susceptible to BILN-2061 (>33-fold) and VX-950 (>10-fold). Biochemical assays using NS3-containing lysates from infected cells confirmed resistance was due to phenotypic changes in the protease. Three NS3 mutations were identified in BILN-2061 resistant viruses: A156G, D168A and D168V. Interestingly, D168A and D168V, but not A156G, were selected in parallel using a genotype 2a replicon. More strikingly, from multiple selections with VX-950, three NS3 mutations were identified in the virus (T54A, A156S, and V102A) but only A156T/V emerged in genotype 2a replicon selections. Of note, resistance mutations selected from virus infection, including T54A, A156S, and A156G, have all been observed in the clinic. We hypothesized that resistant viruses may need to balance fitness and resistance to a greater extent than resistant replicons. To investigate this, each major viral resistance mutation was introduced into the parental J6/JFH-1 virus. Viral kinetic studies demonstrated that the mutant viruses expanded at similar or slightly delayed rates but reached similar peak titers to the parental virus. In conclusion, the HCV infection system is an efficient tool for drug-resistance selections and has advantages to rapidly identify and characterize clinically-relevant resistance mutations.

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In Vitro Selection and Characterization of Hepatitis C Virus Replicons Double or Triple Resistant to Various Non-nucleoside HCV Polymerase Inhibitors

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To prevent, delay or avoid the development of HCV resistance, combination therapies will be necessary. We determined the antiviral efficacy of various combinations of non-nucleoside polymerase inhibitors and the barrier towards resistance development. Short-term antiviral combination assays were performed in replicon containing cells. For resistance selection of mono- and double resistant replicons, a stepwise selection procedure was used. Triple resistant replicons were selected starting with a replicon already resistant to thiophene carboxylic acid (TCA) and the benzofuran HCV-796. All pair wise combinations of elicit an additive anti-HCV effect in short-term antiviral assays. Resistant replicons were selected for three non-nucleoside polymerase inhibitors (TCA, benzimidazole JT-16 and benzofuran HCV-796) and for each pair wise combination. Triple resistant replicons were selected for the following combinations: TCA + HCV-796 + VX-950 (protease inhibitor), TCA + HCV-796 + 2′-C-methylcytidine and TCA + HCV-796 + JT-16. The identified genotype of double and triple resistant replicons is the sum of the single resistance mutations. Cross-resistance selection experiments revealed that combinations of non-nucleoside inhibitors (HCV-796 + TCA and HCV-796 + JT-16)

at concentrations of 5× their EC_{50} (or higher) completely prevented the development of resistance. Our data further support the design of anti-HCV therapy based on combinations of non-nucleoside polymerase inhibitors.

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Identification and Characterization of a Hepatitis C Virus Capsid Assembly Inhibitor

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Hepatitis C virus (HCV) causes chronic infection associated with severe liver disease. About 130 million people are chronically infected worldwide. Current treatment with pegylated interferon-α and ribavirin has limited efficacy and is associated with substantial side effects. Although highly potent HCV enzyme inhibitors are in clinical development, drug-resistance and genotype-specificity may limit these novel therapeutics. Therefore, a combination of drugs targeting different steps of the viral life cycle will likely improve viral response rates and therapy success.

To identify small molecules that interfere with different steps of the HCV replication cycle, we developed a dual reporter gene assay encompassing the complete viral life cycle on 96/384-well format. Upon transfection of Huh-7 Lunet cells stably expressing Gaussia luciferase with an HCV firefly reporter virus, HCV RNA-replication and cell viability/toxicity in the presence of compounds is assessed by a dual luciferase assay. In the second part of the assay, transfer of the culture fluid to naïve cells permits evaluation of the impact on de novo assembly and infectivity of HCV particles. Using a set of known entry, replication and assembly inhibitors we confirmed that the assay is able to identify lead substances affecting either one of these steps and to discriminate molecules with antiviral activity from compounds that merely compromise cell viability.

Screening a large compound library with an in vitro HCV core protein assembly assay, we used our cell based assay to confirm the antiviral activity of compounds in cell culture. The most interesting candidate (PAV-617) was subjected to structure–activity-relationship studies, revealing potent analogs with a strong influence on HCV assembly in cell culture. A proteinase k protection assay indicates that PAV-617 interferes with an early assembly step prior to membrane envelopment of HCV core protein structures. Moreover, we observed an accumulation of core and NS5A proteins around lipid droplets. Together these data suggest that PAV-617 and its derivatives arrest unloading of core protein from lipid droplets thus preventing assembly and release of infectious HCV particles.

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The Requirement of HCV NS3 Oligomerization for RNA Helicase Activity is RNA Substrate Dependent

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Background: The nonstructural protein (NS3) of the hepatitis C virus carries ATPase-coupled helicase activity that requires the presence of a 3′-single stranded RNA overhang. The ability of NS3 to catalyze strand separation of double-stranded RNA is suggested to be essential for HCV replication. The molecular mechanism of RNA unwinding and the role of NS3 oligomerization are currently not well defined.

Methods: Helicase activity was measured with full-length NS3 proteins using a fluorescence based, real-time strand separation assay and RNA substrates of different double strand and single strand lengths. NS3 protein binding to RNA was measured using intrinsic NS3 protein fluorescence quenching (FQ), surface plasmon resonance (SPR) and fluorescence polarization (FP).

Results: NS3 bound with 1:1 stoichiometry to an RNA substrate containing a 21 nt double strand (ds) and 10 nt single strand (ss) overhang, and separated RNA strands efficiently when substrate was in excess. Increasing the ssRNA overhang did not affect the RNA helicase activity in the steady-state under substrate-excess conditions. RNA helicase activity increased with increasing length of ssRNA overhang for substrates with longer double strand RNA regions. However, RNA binding affinity was not affected, consistent with NS3 cooperativity in RNA unwinding, but not RNA binding. NS3 carrying the E291A point mutation was inactive as a helicase, but potently inhibited the RNA helicase activity of wild-type NS3. Trans-dominant inhibition by E291A NS3 was only observed on substrates with long dsRNA regions, whereas RNA helicase activity on ds21-ss10 substrate was not inhibited, suggesting NS3 oligomerization dependence for the unwinding of long, but not short double strand RNA. Inhibitors interfering with RNA binding affected unwinding independently of double strand length.

Conclusion: The mechanism of RNA unwinding by HCV NS3 is substrate dependent. Whereas short double strands could be separated by NS3 monomers, the unwinding of longer double strands require functional NS3 oligomerization. Inhibitor mechanism of action can involve interference with RNA binding.

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Bound Structure and Biochemical Mechanism of Action of BI 201335, a Potent, Non-covalent Inhibitor of HCV NS3-NS4A Protease

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Hepatitis C virus infection is an important cause of liver disease world-wide. This chronic and progressive infection is curable, but currently approved therapies have suboptimal efficacy and significant side effects. Direct-acting antiviral agents have the potential to considerably improve treatment prospects for HCV infected patients. BI 201335 is a direct-acting antiviral agent that targets the viral NS3-NS4A protease. It is a non-covalent, reversible, and competitive inhibitor with K_i values from steady-state kinetics of 2.6 and 2.0 nM, for genotype 1a and 1b respectively, and similar EC_{50} values in cellular replicon assays of 6.5 and 3.0 nM. BI 201335 has advanced to clinical development and is progressing through Phase IIb trials. Here we present the detailed biochemical mechanism of action and the target-bound X-ray crystal structure of this new anti-HCV agent. Pre-steady-state kinetic data for the interaction of BI 201335 with NS3-NS4A were best fit to a slow single-step binding mechanism, with a k_{on} value of approximately $7.5 \times 10^5\ M^{-1}\ s^{-1}$, and a slow off-rate of $4 \times 10^{-4}\ s^{-1}$, giving a dissociation half-life of approximately 30 min. Very similar data were obtained using the NS3 protease domain with an N-terminally fused NS4A peptide and lacking the NS3 helicase domain. Structural studies were carried