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

Juliane Gentzsch[1,*], Clarence R. Hurt[2], Vishwanath R. Lingappa[2], Thomas Pietschmann[1]

[1] *Division of Experimental Virology, TWINCORE, Hannover, Germany*
[2] *Prosetta Bioconformatics, San Francisco, USA*

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

Julie Hang*, Yanli Yang, Junjun Gao, Vincent Leveque, Marie Dinh, Mohammad Hekmat-Nejad, George Stepan, Phil Kobel, Klaus Klumpp

Roche, Palo Alto, USA

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

Christopher T. Lemke*, Songping Zhao, Nathalie Goudreau, Oliver Hucke, Diane Thibeault, Montse Llinàs-Brunet, Peter W. White

Boehringer Ingelheim (Canada) Ltd., Laval, Canada

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