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

out using either this fused NS4A-NS3 protease or the NS3 protease-NS4A peptide complex. BI 201335 was successfully soaked into unliganded NS3 protease-NS4A peptide crystals, and the resulting 1.9 Å crystal structure of the ternary complex clearly shows all interactions of the bound inhibitor with the target. Of particular interest are the C-terminal acid, which interacts with residues of both the catalytic triad and the oxyanion hole, and the quinoline-substituted proline, which induces the formation of a large flat surface partially comprised of catalytic triad residues. This arrangement affects the protonation state of the active site histidine, as demonstrated by NMR. These findings provide detailed insight into the mechanism of action for this promising new candidate for treatment of hepatitis C infection.

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MK-5172, a Novel Macrocyclic Inhibitor of NS3/4a Protease Demonstrates Efficacy Against Viral Resistance in the Chimpanzee Model of Chronic Hepatitis C Virus Infection

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MK-5172 is a potent macrocyclic inhibitor of NS3/4a protease enzyme (IC_{50} < 0.016 nM, replicon IC_{50} = 2 nM) optimized for an improved pharmacokinetic profile and increased potency against resistant virus. As part of its pre-clinical characterization, three chimpanzees chronically infected with HCV were dosed with MK-5172; plasma samples were periodically collected and evaluated for efficacy and viral resistance. Two chimpanzees (gt1a/gt1b) were burdened with high viral load infections ($\sim 10^6$ IU/ml) while a third sustained a modest viral load of 10^4 IU/ml that was homogenous for R155K virus. All three chimpanzees had previously undergone experimental treatment with the first generation protease inhibitor MK-7009. Both high viral load chimps experienced rapid, robust reductions in viral load of 4 and 5 logs, respectively. The R155K infected chimpanzee experienced a two log viral load reduction within 2 days and remained similarly suppressed through the duration of the dosing period. All three demonstrated viral load reductions one log or greater compared to their responses to MK-7009 at equal or higher doses. Sequence analysis of NS3/4a demonstrated no emergence of additional homogenous resistance or genetic polymorphisms. MK-5172 possesses an attractive pharmacokinetic and potency profile, and demonstrates suppression of resistant virus in vivo. Clinical studies with MK-5172 are currently in progress.

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Oral Session 3: Retroviruses and Herpesviruses

Chairs: Rhonda Cardin, Ph.D. and Masanori Baba, Ph.D., 9:00–11:30 am, Grand A

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Identification of a N-linked Glycan on HIV-1 gp120 that is Indispensable for Viral Entry

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Carbohydrate-binding agents (CBAs) inhibit HIV-1 entry into susceptible cells by binding to the N-linked glycans that make up the protective glycan shield on the viral envelope. Under increasing pressure of the CBAs, HIV-1 deletes a variety of N-linked envelope glycans to escape drug pressure. More than 50 virus strains were already selected under CBA pressure, but none of them showed glycan deletions at the N239, N260, N354 and N461 amino acid positions of gp120. To reveal why certain N-glycosylation sites were so far not deleted under CBA pressure in HIV-1 infected cell cultures, the plasmid pBlue-Env which encodes the HIV-1 env gene was used to generate mutant virus envelope gp120 with disrupted glycosylation sites at positions N239, N260, N354 and N461, where Asn was replaced by Gln. A variety of mutant viruses were constructed by env chimeric virus technology with the pNL4.3-Denv-EGFP plasmid and subsequent transfection of 293T cells. Neither the mutant N260Q/A nor the S262C/A gp120 HIV-1 strains in which the 260NGS262 glycosylation motif was destroyed were able to infect the U87.CD4.CCR5.CXCR4 cells. The replication capacity of the 3 other mutant monodeglycosylated gp120 virus strains (N239Q, N354Q and N461Q) was reduced but not annihilated. Whereas 293T cells expressing the HIV-1 wild-type gp120 envelope were able to induce syncytia when cocultured with U87.CD4.CCR5.CXCR4 cells, giant cell formation was not observed when 293T cells expressed the mutant N260Q HIV-1 gp120 envelope. This loss of infection potential and capacity to form giant cells in co-cultures was not due to a lethal defect in Env processing. Instead, the mutant N260Q gp120 virus displayed a significantly decreased CD4 binding ability. In conclusion, deletion of the N-linked glycan at amino acid position N260 of HIV-1 gp120 results in the complete loss of infectivity and capacity to induce giant cell formation. This is probably caused by a significant lower CD4-binding compared to wild-type virus. Since N260 is a highly conserved glycosylation site among the different clades of HIV-1, the crucial role of the N-linked glycan at amino acid position 260 of HIV-1 gp120 for viral infectivity makes it an interesting target for antiviral therapy or vaccine development against HIV-1.

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