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Discovery of a Novel HCV Helicase Inhibitor by a De Novo Drug Design Approach

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Structure-based drug design methods utilize knowledge of a three-dimensional structure of an enzyme/receptor to develop small molecules able to bind to the desired target, generating a specific biological response. These computer-based methodologies are now becoming an integral part of the drug discovery process and, although the principles of molecular recognition are far from being completely understood, some marketed compounds (i.e. influenza neuraminidase inhibitors and HIV protease inhibitors) have been developed with a successful application of structure-based design techniques.

In this presentation we are reporting a successful application of a computer-aided design approach to identify and synthetize a series of novel HCV helicase inhibitors. Initially a putative binding site was identified on the enzyme surface, then a de novo drug design software package was used to generate an initial set of structures that could potentially bind to it. A further structure refinement was carried out by docking a series of virtual libraries derived from the de novo procedure. The best structure identified in silico (AB100) was then prepared and it exhibits a submicromolar inhibition of the HCV helicase. The results of the replicon assay as well as the enzymatic assay for AB100 and a series of related analogues will be also presented.

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PG 301029 Inhibits HCV Replication Through a Novel Late Stage Mechanism of Action

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PG 301029 was identified from a large structure–activity relationship evaluation as a highly effective small molecule inhibitor of the replication of the hepatitis C virus. Primary screening was performed using bovine viral diarrhea virus (BVDV), and the activity was confirmed using the HCV replicon system. Subsequent IND-directed development of the compound has demonstrated a novel mechanism of antiviral action of PG 301029 and the possibility of using the compound in combination with current therapies. PG 301029 exhibited an EC_{50} value of 0.65 μg/mL against BVDV, was 100–200-fold less toxic than ribavirin to cells infected with BVDV, and was nontoxic to fresh human hepatocytes at the highest concentration tested (316 μg/mL). Mechanism of action studies revealed that PG 301029 inhibited BVDV replication through suppression of viral RNA synthesis that was not related to inhibition of virus entry, translation initiation, or inhibition of the NS2/3 viral proteinase. Additionally, in combination anti-BVDV assays, the addition of PG 301029 to ribavirin or ribavirin plus interferon yielded synergistic anti-HCV activity and also resulted in a reduction of the toxicity of ribavirin. PG 301029 is efficacious in an HCV replicon system, yielding an EC_{50} concentration of 0.38 μg/mL. We hypothesize that PG 301029 inhibits HCV replication through a novel late stage mechanism of action which results in significant reductions in the accumulation of viral RNA in infected cells. Confirmation of the antiviral mechanism of action has been performed using the HCV replicon system as well as from the selection and characterization of PG 301029-resistant HCV replicons. Additionally we have examined the effect of PG 301029 on HCV protein processing and stability, on viral RNA synthesis and stability, and on the differential expression of IFN-regulated genes in treated Huh-7 cells and Huh-7 cells transfected with HCV replicons. Results of *in vitro* combination studies for the inhibition of HCV replicons by PG 301029 with ribavirin and IFN-α will also be presented. PG 301029 exhibits a novel antiviral mechanism of action distinct from that of existing anti-HCV therapies and clinical use of PG 301029 in combination with existing agents may provide significant therapeutic benefits.

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Different Incorporation Efficiencies for Nucleotide Analogs During HCV Polymerase RNA Synthesis Initiation and Elongation Phases

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Background: Most antiviral nucleotide analogs function as alternative substrates for the viral polymerase and result in chain termination after incorporation. Potential differences in incorporation efficiency relative to natural NTPs and the effect of the S282T resistance mutation during initiation and elongation phases of RNA synthesis by HCV polymerase are not well understood.

Methods: Gel-based assays were developed to determine the kinetic parameters of nucleotide incorporation by NS5B wild-type and S282T mutant NS5B at initiation or elongation phase. Incorporation kinetics were determined for nucleotide analogs including development candidates R1479-TP, NM107-TP, RO-9187-TP and RO-0622-TP.

Results: The elongation complex of NS5B showed significantly higher incorporation efficiency of the natural CTP substrate as compared to the initiation complex. The elongation complex was not inhibited by non-nucleoside inhibitors of HCV polymerase or by heparin. In contrast, nucleotide analogs were incorporated during initiation and elongation phases of RNA synthesis and could inhibit RNA synthesis during both phases. Among the CTP analogs tested, 4′-azido-CTP (R1479-TP) was the most efficient alternative substrate for NS5B during both initiation and

elongation. The comparative analysis of incorporation kinetics of other cytidine analogs (e.g. NM107-TP) and deoxycytidine analogs (RO-9187-TP, RO0622-TP) suggest important contributions of 2′- and 4′-substituents for interaction with NS5B, as well as differences in interaction between initiation and elongation complexes. The results obtained with the S282T mutant NS5B suggest differential effects of this mutation on nucleotide analog incorporation efficiency during initiation and elongation as well as different impact of 2′- and 4′-substitutions.
Conclusions: Nucleoside analogs were incorporated by NS5B during initiation and elongation phases of RNA synthesis, albeit with significantly different efficiencies. The 2′- and 4′- positions can differentially affect incorporation efficiency by wild-type and S282T mutant NS5B.

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HCV RNA Helicase Requirement for NS3 Oligomerization is Substrate-dependent: Characterization of RNA Substrates with Different Double Strand Lengths and RNA Binding-dependent Inhibition

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Background: HCV NS3 carries ATPase-coupled helicase activity, dependent on the presence of a 3′-single-stranded RNA overhang. NS3 catalyzed strand separation of double-stranded RNA is essential for HCV replication. The molecular mechanism of RNA unwinding and the role of NS3 oligomerization are 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), fluorescence polarization (FP), and surface plasmon resonance (SPR) with biotinylated RNA.
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 was separating RNA strands efficiently under these conditions. Increasing the ssRNA overhang did not affect helicase activity, whereas shortening of ssRNA length led to reduced RNA binding affinity and helicase activity, consistent with ssRNA overhang requirement for functional NS3 binding. 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. The NS3 E291A mutant was inactive as a helicase, but potently inhibited 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 RNA double strands. Inhibitors interfering with RNA binding affected unwinding independent of double strand length.
Conclusion: The mechanism of RNA unwinding by HCV NS3 is substrate-dependent. Short double strands could be separated by NS3 monomers, but longer double strands require functional NS3 oligomerization. Inhibitor mechanism of action can involve interference with RNA binding.

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Evaluation of MK-7009, A Novel Macrocyclic Inhibitor of NS3/4A Protease, in the Chimpanzee Model of Chronic Hepatitis C Virus Infection

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MK-7009 is a potent macrocyclic inhibitor of NS3/4a (replicon $EC_{50} = 3.5$ nM) currently in clinical development. As part of its pre-clinical characterization, chimpanzees chronically infected with HCV were dosed with MK-7009 and evaluated for efficacy and viral resistance. Two chimpanzees harboring genotype 1a chronic infections and with initial viral loads >6 logs were dosed orally for 7 days at 5 mpk b.i.d. Both experienced rapid viral load reductions of >5 logs before eventually relapsing to baseline levels following cessation of dosing. Viral RNA was isolated from plasma samples that had been collected periodically throughout the study. NS3/4a was cloned by RT-PCR and analyzed with a variety of techniques including sequencing, an allele-specific Taqman assay (detection limit 1/5000 copies), and phenotypic analysis of viral NS3/4a sequences derived from the plasma. Population sequencing demonstrated that R155K virus emerged as the principle circulating virus for both chimpanzees. Phenotypic analysis of viral NS3/4a sequences showed that R155K conferred >100-fold loss of susceptibility to the inhibitor. Using an allele-specific Taqman assay sensitive to the R155K mutant, R155K virus was shown to pre-exist in the circulating viral population of one chimpanzee at less than 1% of the total population. By the second day of dosing it encompassed virtually the entire circulating population. Its replication, however, showed partial suppression and continued to decline with further dosing. The R155K variant emerged as the predominant species early in the relapse population of both chimpanzees, but was slowly replaced by wild-type over the course of several months. Conclusion: a pre-existing resistant virus was enriched during initial exposure to MK-7009 but continued dosing also suppressed replication of this mutant virus. The long-term persistence of R155K virus demonstrated that its replication was not severely impaired. The results demonstrate the importance of resistance monitoring during direct antiviral therapy to treat HCV infection, and suggest that inhibitor potency evaluations against potentially prevalent mutants is warranted.

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