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Development and Validation of a High Throughput Screen for Inhibitors of Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV) is the most common cause of bronchiolitis and pneumonia among infants and children under 1 year of age, but severe lower respiratory tract disease may occur at any age, especially among the elderly or among those with compromised cardiac, pulmonary, or immune systems. The existing therapies for the acute infection are ribavirin, which has inconsistent clinical results, and the prophylactic humanized monoclonal antibody (Synagis® from MedImmune) that is expensive and limited to use in high risk pediatric patients. Thus, there is a critical need to discover novel antiviral drugs to supplement existing chemotherapeutics. To meet this need, we have developed a high-throughput screen (HTS) that allows for the identification of potential inhibitors of RSV from large compound libraries. This cell-based assay measures the inhibition of RSV strain long-induced cytopathic effect (CPE) in HEp-2 cells 72 h post-infection using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). The assay is sensitive and robust, with Z values > 0.8, signal to background, S/B > 22, and signal to noise, S/N > 5. Various parameters were optimized and validated including cell density, viral concentration, DMSO tolerance for compound dilution, incubation time for virus-induced CPE and effective control drug concentration. Additional parameters, such as day-to-day assay variability, reagent and read stability, edge effects, and IC50 stability were also examined during validation. We are using this assay to screen chemical libraries and report here our findings from these screens.

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Development of Intergenotypic Chimeric Replicons for Broad-spectrum Antiviral Activity Characterization of Hepatitis C Virus Polymerase Inhibitors

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The identification of hepatitis C virus (HCV) inhibitors with broad-spectrum activity has been hampered by the limited number of HCV replicons that replicate efficiently in cell culture. To date, only genotype (gt) 1a, 1b, and 2a subgenomic replicons are available. To address the need for broad-spectrum activity characterization of HCV NS5B polymerase inhibitors, we created a panel of intergenotypic chimeric replicons containing NS5B sequences from gt 2b, 3a, 4a, 5a, and 6a HCV isolates.

Viral RNA extracted from non-gt1 HCV patient plasma was subjected to reverse transcription. The NS5B region was amplified by nested PCR and cloned into the corresponding region of the gt 1b (Con-1) subgenomic reporter replicon by SOEing PCR. Replication fitness was determined based on reporter activity and colony formation efficiency following electroporation of chimeric replicon RNAs into Huh7.5 cells. Stable replicon cell lines were generated for in vitro antiviral activity determination of HCV inhibitors. Inhibition of HCV RNA replication was measured, after a 3-day incubation with compounds, using the reporter activity of the stable cell lines as an endpoint.

Replication fitness of the gt 4a NS5B chimeric replicon was comparable to that of the gt 1b replicon, whereas introduction of the gt 2b, 3a, 5a, and 6a NS5B sequences reduced replication efficiency by 6–100-fold compared to the gt 1b replicon. In antiviral assays, nonnucleoside polymerase inhibitors (NNPI) that bind to either the thumb base or the primer grip regions of NS5B displayed a 2–3 log decrease in antiviral activity against the gt 2b, 3a, 4a, 5a, and 6a NS5B chimeric replicons when compared to the gt 1a and 1b replicons. Evaluation of the antiviral activity for an NNPI that binds to the thumb tip site demonstrated a comparable reduction in activity against the gt 2b chimeric replicon, and smaller reductions against gt 3a, 4a, and 5a chimeras when compared to the gt 1a and 1b replicons.

In conclusion, evaluation of HCV polymerase inhibitors against intergenotypic chimeric replicons showed differences in activity spectrum for inhibitors that target different regions of the enzyme. Our study demonstrates the utility of chimeric replicons for broad-spectrum activity determination of HCV inhibitors.

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shRNAs Targeting Hepatitis C: Effects of Sequence and Structural Features, and Comparison with siRNA

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Hepatitis C virus (HCV) is a leading cause of liver cirrhosis and hepatocellular carcinoma worldwide. Currently available treatment options are of limited efficacy, and there is an urgent need for development of alternative therapies. RNA interference (RNAi) is a natural mechanism by which small interfering RNA (siRNA) or short hairpin RNA (shRNA) can mediate degradation of a target RNA molecule in a sequence-specific manner. In this study, we screened in vitro-transcribed 25-bp shRNAs targeting the internal ribosome entry site (IRES) of HCV for the ability to inhibit IRES-driven gene expression in cultured cells. We identified a 44-nt region at the 3′-end of the IRES within which all shRNAs efficiently inhibited expression of an IRES-linked reporter gene. Subsequent scans within this region with 19 bp shRNAs identified even more potent molecules, providing effec-

tive inhibition at concentrations of 0.1 nM. Experiments varying features of the shRNA design showed that, for 25 bp shRNAs, neither the size of the loop (4–10 nt) nor the sequence or pairing status of the ends affects activity, whereas in the case of 19-bp shRNAs, larger loops and the presence of a 3′-UU overhang increase efficacy. A comparison of shRNAs and siRNAs targeting the same sequence revealed that shRNAs were of comparable or greater potency than the corresponding siRNAs. Anti-HCV activity was confirmed with HCV subgenomic replicons in a human hepatocyte line. The results indicate that shRNAs, which can be prepared by either transcription or chemical synthesis, may be effective agents for the control of HCV.

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Synthesis and Anti-HBV Activity of 7-Deaza-Neplanocin A Analogs

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Four anti-HBV nucleosides (Lamivudine, Adefovir dipivoxil, Entecavir and Telbivudine) have been approved by the US FDA for the treatment of chronic HBV infection. In addition to these drugs, several other nucleosides such as Clevudine (L-FMAU), Valtorcitabine (LdC) and Tenofovir are currently under various stages of clinical evaluation. However, a significant number of patients develop drug resistance during the long-term use of these agents. Thus, there is a critical need to continue discovering and developing safe and effective novel anti-HBV agents to cope with the drawbacks of the current agents.

As part of our antiviral drug discovery program, it was of interest to synthesize less toxic neplanocin A analogs as potential anti-HBV agents. For the synthesis of the analogs, we utilized a key cyclopentenyl carbocyclic intermediate, which was previously developed in our laboratory. From the synthesis, we prepared 10 7-deaza-neplanocin analogs.

Among the 7-deaza naplanocin A analogs, two analogs exhibited significant anti-HBV activity with EC_{50} values of 0.43 and 0.32 μM, respectively based on extracellular HBV virions with negligible cytotoxicity ($CC_{50} > 300$ μM). In addition, these analogues also showed significant anti-HBV activity against variety of clinically relevant Lamivudine-resistant mutants.

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A Novel Class of Amphipathic DNA Polymers Inhibits Hepatitis C Virus Infection by Blocking Viral Entry

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Long (>30 base) phosphorothioate oligonucleotides (PS-ONs) are novel amphipathic polymers that display a sequence-independent antiviral activity against numerous type 1 enveloped viruses. The amphipathic nature of these long PS-ONs targets them to the amphipathic alpha helical "hinge" domains of fusion proteins and inhibits viral entry by blocking virus-cell fusion. The antiviral activity of these molecules in viruses with type I or II fusion mechanisms suggest that structural similarities exist between type I and type II fusion proteins. The aim of this study was to assess the ability of long PS-ONs to inhibit HCV infection by blocking viral entry. Huh7.5 cells were infected with the HCV-containing culture medium (HCVcc) and treated with various PS-ON analogs to assess their inhibitory activity. The antiviral mechanism of action of these compounds was further examined in viral binding and entry assays with HCV-like particles (HCV-LPs) and HCV pseudovirus (HCVpp), respectively and in HCV replicon systems. PS-ONs displayed a similar size dependent antiviral activity, plateauing with 40mer and longer PS-ONs. These compounds potently inhibited HCV infection in both the HCVcc and HCVpp systems with IC_{50} in the range of 10–100 nM and were equally active against HCVpp of various genotypes. Several chemical modifications of these polymers which eliminated their amphipathic character also rendered them ineffective against HCV infection. Active PS-ONs had no effect on viral replication in the NK5.1 and JFH-1 replicon systems or binding of HCV-LPs to Hep3B or Huh7.5 cells but did prevent internalization of HCVpp virions into Hep3B cells, indicating that the target of inhibition by PS-ONs is at the post-binding, cell entry step. Thus PS-ONs (amphipathic DNA polymers) are a promising new class of antiviral compounds that inhibit HCV fusion and entry. The similar chemical requirements on the PS-ON structure (size and amphipathic character) for antiviral activity in both HIV-1 and HCV suggest that the entry mechanisms of HIV-1 and HCV are similar.

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