differ in glycosylation pattern of viral HA possessed markedly reduced drug susceptibility against NAI in cell culture based assays.

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Activation of GS-7340 and Other Tenofovir Phosphonoamidate Prodrugs by Human Proteases

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GS-7340 is an isopropylalaninyl phenyl ester prodrug of a nucleotide HIV reverse transcriptase inhibitor tenofovir (TFV; 9-[(2-phosphonomethoxy)propyl]adenine) exhibiting potent anti-HIV activity and enhanced ability to deliver parent TFV into peripheral blood mononuclear cells (PBMCs) in vivo. The present study focuses on the intracellular metabolism of GS-7340 and its activation by a variety of cellular hydrolytic enzymes. Incubation of human PBMCs in the presence of GS-7340 indicate that the prodrug is more efficiently hydrolyzed to an intermediate TFV-alanine conjugate (Met X) in quiescent PBMCs compared to activated cells. In contrast, the conversion of Met X to TFV and subsequent phosphorylation to TFV-diphosphate occur more rapidly in activated PBMCs. The activity of GS-7340 hydrolase producing Met X in PBMCs is primarily localized to lysosomes and is sensitive to inhibitors of serine hydrolases. Cathepsin A, a lysosomal serine protease has recently been identified as the primary enzyme activating GS-7340 in human PBMCs. Result from the present study indicate that in addition to cathepsin A, a variety of serine and cysteine proteases cleave GS-7340 and other phosphonoamidate prodrugs of TFV. The substrate preferences displayed by the tested proteases towards a series of TFV amidate prodrugs is nearly identical to their relative activities displayed against peptide substrates, indicating that GS-7340 and other amidate derivatives can be considered peptidomimetic prodrugs of TFV.

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NIM811, A Cyclophilin Inhibitor, and NM107, An HCV Polymerase Inhibitor, Synergistically Inhibits HCV Replication and Suppresses the Emergence of Resistance In Vitro

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More effective and better tolerated therapies are needed for chronic hepatitis C which affects 170 million people worldwide. Because of the high heterogeneity and mutation rate of hepatitis C virus (HCV), future therapies are likely to consist of multiple drugs to maximize antiviral efficacy and to prevent resistance. We are taking a two-prong approach to develop novel therapeutic agents for HCV. The first strategy is to target viral proteins such as the NS5B RNA polymerase directly. One such inhibitor, NM283 (valopicitabine), is currently in Phase II clinical trials. The second and a complementary strategy is to target host factors that are also essential for viral replication. NIM811, a cyclophilin inhibitor with potent in vitro antiviral activities, represents such an approach and is under clinical investigation in HCV patients. Here, the combination of NIM811 and NM107 (the active moiety of NM283) was evaluated in vitro using the HCV replicon model as the first step to explore the possibility of using such a combination in patients. HCV replicon cells were treated with various concentrations of the two compounds either alone or in combination. There was a concentration- and time-dependent inhibition of HCV replicon with NIM811 and/or NM107. Importantly, the combination always led to a stronger antiviral effect than either agent alone with no significant increase of cytotoxicity. Moreover, the effect of combination was determined to be synergistic as analyzed in a mathematic model. In addition, drug-resistant clones were generated, and there was no cross-resistance between these two inhibitors of different mechanisms. Furthermore, the frequencies of resistance were determined with the compounds at various concentrations. The barrier to resistance was greatly increased when NIM811 and NM107 were used in combination. In summary, these in vitro results illustrate the significant advantages of combination therapies and warrant exploration of this specific combination in further studies.

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Identification of Novel Low Molecular Weight HIV-1 gp41 Fusion Inhibitors Using A New Quantitative High Throughput Fluorescence Intensity Assay

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A new high throughput screening assay for HIV-1 gp41 inhibitors has yielded novel low molecular weight fusion inhibitors from a peptidomimetic library. Both the assay and the new compounds are described. The assay is performed by mixing two designed peptides with compounds arrayed in multi-well plates, and measuring fluorescence intensity. It can be readily applied to screen large chemical databases for identification of HIV-1 fusion inhibitors. Inhibitors can be detected quantitatively from the assay in three simple steps: (1) a high throughput screen to identify possible positive hits by fluorescence intensity enhancement; (2) a control high throughput screen to eliminate false positives; (3) serial dilution of true positive hits to obtain high throughput dose–response curves for determination of inhibition constants (K_i). The HTS assay has a Z' factor of 0.88 and can rank order inhibitors at 10 μM concentration with K_i's in the range 0.2–30 μM, an ideal range for drug discovery. The assay was validated using known gp41 inhibitors. The applicability

of the assay was verified by a screen of a small peptidomimetic compound library, with the discovery of three novel HIV-1 gp41 inhibitors. The best hit has a molecular weight below 500, binds with a K_i of 1.2 μM, and inhibits syncytium formation at micromolar concentration in vitro.

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Development of ATP/Luminescence Assays For Profiling Compounds Against A Panel of Positive-Strand RNA Viruses

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We designed an ATP-based assay platform to profile antiviral compounds against a number of positive strand RNA viruses including yellow fever virus (flavivirus), West Nile virus (flavivirus), Coxsakie B virus (picornavirus) and sindbis virus (alphavirus). This assay platform is based upon the bioluminescent measurement of ATP in metabolically active cells. Antiviral efficacy was determined by measuring the ATP level in cells that were protected from the viral cytopathic effect (CPE) by the presence of antiviral reference or test compounds.

The bioluminescent ATP-detection method utilizes luciferase which catalyses the formation of light from ATP and luciferin. The emitted light intensity is linearly related to the ATP concentration in live cells and was measured using a ViewLux apparatus (Wallace). In these assays, Vero cells were seeded in 96-well plates in the presence of various concentrations of test compounds and infected with different viruses. The assay is homogeneous (mix and measure) and amenable for high-throughput screening.

The ATP/luminescence assay parameters were optimized and the assays were validated using different reference compounds to determine intra- and inter-assay reproducibilities. The signal to noise ratios for yellow fever virus and West Nile virus were 7.5 and 36, respectively. This compares favorably to the signal to noise ratio of only 1.5 in the neutral red dye uptake assay for yellow fever virus, an alternative readout for CPE inhibition. For Coxsakie B and sindbis virus, the signal to noise ratios were 40 and 50, respectively.

In conclusion, we have validated the ATP/luminescence assay for profiling antiviral compounds against yellow fever virus, West Nile virus, sindbis virus and Coxsackie B virus, representing three virus families. These assays are robust, high-throughput, reproducible and give much better signal to noise ratios than that of dye uptake assays.

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Discovery of Two Novel Classes of Inhibitors of Hepatitis C Virus (HCV) Replication utilizing a Dicistronic Reporter HCV Replicon High Throughput Assay

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Background: We report the discovery of two novel classes of HCV replication inhibitors using the HCV subgenomic replicon system in a high throughput screen (HTS).

Methods: The HCV genotype 1b subgenomic replicon was modified to include a humanized Renilla luciferase (hRLuc) followed by the antibiotic resistance gene neo^R. Antiviral activity was monitored using the hRLuc gene as an endpoint. A stable cell line was obtained by the introduction of the modified subgenomic replicon into Huh-7 cells and subsequent clonal selection. The firefly luciferase (FLuc) gene was subsequently introduced into the chromosome of the replicon-containing cell line to monitor compound cytotoxicity. Robustness of both signals from this dual reporter cell line has provided the flexibility of screening in the HTS or low-throughput, multidose assay formats as well the simplicity of obtaining antiviral activity and cytotoxicity data in a single well.

Results: Greater than 1.5 million compounds were screened using this dual reporter HCV replicon cell with a calculated Z' value of 0.8 indicating low variability of both reporter signals. Approximately, 18,000 confirmed hits were generated with a confirmation rate of 40%. Two novel classes of compounds were identified from this screening effort. One series of compounds contain hydroxamic acid residues and have 50% effective concentrations (EC_{50}) values ranging from 50 to 1500 nM and therapeutic indices (TIs) ranging from 19 to 1980. The second series of compounds contain a urea-thiazole motif and have EC_{50} values ranging from 11 to 16,000 nM and TIs ranging from 1 to >1230. Efforts are currently underway to investigate the potential molecular target(s) for these two series of compounds.

Conclusions: We describe the construction of a modified HCV subgenomic replicon as well as the generation of a replicon cell line capable of detecting antiviral activity and cytotoxicity simultaneously in a single well. We will also report the identification, in vitro antiviral activity and initial structure activity relationship of two classes of small molecule inhibitors of HCV replication from an HTS screen.

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