prophylaxis and therapy of respiratory virus infections cause by IAV or HRV.

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Development of Resistance to the Natural HIV-1 Entry Virus Inhibitory Peptide (VIRIP)

Emmanuel Gonzalez*, Maria Pau Mena, Mercedes Armand-Ugon, Ester Ballana, Bonaventura Clotet, Jose Esté *IrsiCaixa, Badalona, Spain*

The virus inhibitory peptide (virip) was identified as a component of human hemofiltrate and shown to have anti-HIV activity through the inhibition of HIV-1 gp41-dependent fusion. We confirmed the anti-HIV activity of virip and the optimized virip-derived peptide vir353 in lymphoid cells. Virip and vir-353 showed a dose-dependent activity with 50% effective concentrations of 16 and 0.7 mM respectively and a time of addition experiment showed that virip and vir353 target a time/site of action that corresponds to gp41-dependent fusion. Sequential passage of HIV-1 NL4-3 in lymphoid MT-4 cells in the presence of increasing concentration of different anti-HIV drugs led to the generations of virus resistant to nevirapine (10 days), the entry inhibitor BMS-155 (30 days), the fusion inhibitors, enfuvirtide (90 days), sifuvirtide (180 days) and vir-353 (260 days) suggesting a high genetic resistance for the virip-related compound. The resulting vir-353 resistant virus was completely cross-resistant (>200-fold) to virip but remained sensitive to the fusion inhibitors enfuvirtide and C34 as well as other HIV inhibitors targeting virus entry (AMD3100) or reverse transcriptase (AZT, nevirapine). Recombination of gp41 of the virip resistant virus into a wild-type HxB2 backbone partially recovered the resistant phenotype but both resistant gp120 and gp41 were necessary to recover full resistance to virip. Mutations were found in both gp120 and gp41 of the virip-resistant virus. However, no mutations were found in the fusion peptide of gp41 the alleged target of virip. The time needed to generate a virip resistant virus and the position of mutations found suggest that virip may target an essential part of gp41 and highlight possible interactions between gp41 and gp120 required during the fusion process.

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Single-dose Intranasal Delivery with DEF201 (Adenovirus Vectored Mouse Interferon-α) Protects Against Phlebovirus and Sars Coronavirus Challenge

Brian Gowen[1,*], Dale Barnard[1], Min-Hui Wong[1], Deanna Larson[1], Josh Wu[2], Jane Ennis[3], John Morrey[1], Jeffery Turner[3] [1] *Institute for Antiviral Research and Department of Animal, Dairy, and Veterinary Sciences, Logan, USA* [2] *Defence R&D Canada – Suffield, Medicine Hat, Canada* [3] *Defyrus Inc., Toronto, Canada*

Interferon (IFN)-α is an effective and safe recombinant human protein with broad clinical appeal. While recombinant IFN-α has great therapeutic value, its utility for biodefense is hindered by its short *in vivo* half-life and costly production. Here we describe the use of Ad5-mIFN-α (DEF201) to address these limitations as a prophylactic countermeasure in two murine viral challenge models, Punta Toro virus (PTV; *Bunyaviridae*, *Phlebovirus*) and SARS coronavirus (CoV). Significant protection ($p < 0.001$) against PTV and SARS-CoV infections was observed in mice from a single dose of DEF201 administered 1 day to 3 weeks prior to challenge. DEF201 was delivered intranasally to stimulate mucosal immunity at the probable site of infection and bypass any preexisting immunity. Intramuscular inoculation with DEF201 rapidly increased (~3 h) IFN-α concentrations in unchallenged mice and persisted for extended periods of time. In contrast, a control Ad5 construct elicited only small amounts of IFN-α that were short-lived. Studies investigating the kinetics of mucosal and systemic IFN-α levels following intranasal administration of DEF201 are underway. Effective medical countermeasures that are highly stable, easily administered, and elicit long lasting protective immunity are much needed. The DEF201 technology has the potential to address all of these issues and serves as a broad-spectrum approach to enhance host defense against a number of viral pathogens.

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Evaluation of the Contribution of Amantadine, Ribavirin, and Oseltamivir in a Triple Combination Antiviral Drug (TCAD) Regimen to Suppressing the Emergence of Resistance using a Novel Quantitative Approach

Justin Hoopes[1,*], Minh Le[3], Elizabeth Driebe[2], Erin Kelley[2], David Engelthaler[2], Amy Patick[3], Jack Nguyen[3] [1] *Utah State University, Logan, USA* [2] *TGen North, Flagstaff, USA* [3] *Adamas Pharmaceuticals, Emeryville, USA*

Background: We have previously demonstrated that a triple combination antiviral drug (TCAD) regimen comprised of amantadine, ribavirin, and oseltamivir carboxylate was highly active and synergistic against susceptible and resistant influenza A viruses *in vitro*. To determine the contribution of each drug in TCAD to preventing the emergence of resistance, we have developed a novel assay to quantify the development of resistance following serial passage under drug pressure.

Methods: MDCK cells in 96-well plates were infected with influenza A/Hawaii/31/2007 (H1N1) in the presence of a clinically achievable, fixed concentration of two drugs alone, or in triple combination with varying concentrations of the third drug, using 12 replicates for each condition. Following 5 serial passages, the percentage of wells for each condition having virus breakthrough (>50% cytopathic effect) and the presence of resistance-associated mutations (>1% total population of variants bearing the V27A, A30T, and S31N substitution in M2, and the H274Y substitution in neuraminidase) was determined by neutral red staining and mismatch amplification mutational analysis, respectively.

Results: Treatment of infected cells with any double combination resulted in virus breakthrough in up to 12 of 12 wells (100%) and virus resistance in up to 10 of 11 wells (91%). Addition of each third drug (TCAD) resulted in concentration dependent reductions in the percentage of wells with virus breakthrough and virus resistance. Importantly, the contribution of each drug in preventing the emergence of resistance was shown by a statistically greater ($P < 0.05$) reduction in virus breakthrough and/or emergence of influenza resistant variants compared to all double combinations at clinically achievable concentrations.

Conclusion: These data demonstrate that all three drugs in the TCAD regimen contributed to prevent the emergence of resistance, as determined by virus breakthrough and/or the presence of resistance-associated mutations.

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Orally Bioavailable Anti-HBV Dinucleotide Acyloxyalkyl Prodrugs

Radhakrishnan Iyer[1,*], John Coughlin[1], Cassandra Kirk[1], Seetharamaiyer Padmanabhan[1], Brent Korba[2], Kathleen O'Loughlin[3], Carol Green[3], Jon Mirsalis[3], John Morrey[4] [1] *Spring Bank Pharmaceuticals, Inc., Milford, USA* [2] *Georgetown University, Rockville, USA* [3] *SRI International, Menlo Park, USA* [4] *Utah State University, Logan, USA*

We have previously reported that phosphorothioate di-, and trinucleotides are a new class of anti-HBV compounds with potent activity in vitro and in vivo. We report here the evaluation of acyloxyalkylester prodrugs 2 and 3, derived from the anti-HBV dinucleotide [R_p,S_p]-3′-dA-ps-$U_{2'OMe}$ (**1**). The bioreversibility studies of 2 and 3 – using mouse, rabbit, and human serum – revealed that each isomer of **2** and **3** underwent stereospecific conversion to the active **1** at almost equal rates. The anti-HBV evaluation of **3** in the HepG2.2.15 cell lines revealed that the compound had antiviral potency similar to that of ADV, and antiviral activity against all tested Lamivudine and ADV-resistant mutants. The cytotoxicity evaluation using MDBK, Vero, and HFF cell lines showed that both prodrugs **2** and **3** had $CC_{50} > 1000$ μm indicating a high safety profile. The compounds **2** and **3** displayed high stability in simulated gastric fluid with $t_{1/2} > 1$ h. The pharmacological bioavailability studies of orally administered 2 and 3 in Swiss Webster mice revealed the presence of the dinucleotide 1 in liver. Biodistribution studies of ^{35}S-labeled-**3** in Sprague–Dawley rats revealed that the ratio of liver to plasma concentration of radioactivity was as high as 2.9 (iv route) and 3.9 (po route). The initial pharmacodynamic evaluations of **2**, and **3** at high doses of 300 and 400 mg/kg/day in the HBV transgenic mouse model showed that both compounds had strong anti-HBV activity. Dose-ranging studies of **3** at 1, 5, 10, and 100 mg/kg revealed a dose-dependent reduction of liver HBV DNA as determined by Southern blot analysis and RT-PCR. In summary, the dinucleotide prodrugs 2 and 3 represent the first-in-class orally bioavailable antiviral agents against HBV.

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Activation of Retinoic Acid Inducible Gene (RIG-I) by Nucleotide Analogs: A Potential Novel Mechanism for Antiviral Discovery

Radhakrishnan Iyer[1,*], John Coughlin[1], Seetharamaiyer Padmanabhan[1], Brent Korba[2], Sua Myong[3] [1] *Spring Bank Pharmaceuticals, Inc., Milford, USA* [2] *Georgetown University, Rockville, USA* [3] *University of Illinois, Urbana-Champaign, USA*

Retinoic acid inducible gene (RIG-I) is a host cellular cytosolic protein, that acts as a viral sensor for recognition of double-stranded viral RNA, and stimulates type I interferon production thereby inhibiting viral replication and suppressing cellular permissiveness for virus infection. Using a novel cell-free assay, we have discovered that chemically modified short oligonucleotides induced rapid translocation (shuttling) of RIG-I on a double-stranded RNA (dsRNA) template. The shuttling of RIG-I on dsRNA may have two consequences in vivo: (a) the oligonucleotide-induced shuttling can cause prolonged occupancy of RIG-I on viral RNA and interfere with viral protein/nucleic acid interaction thereby inhibiting viral nucleic acid replication/translation; (b) rapid translocation of RIG-I can activate the downstream mitochondrial antiviral signaling pathway (MAVS) by efficiently exposing the caspase activation and recruitment domains (CARDs) of RIG-I for subsequent ubiquitination and interaction with MAVS to coordinate an immune or apoptotic response. Since RIG-I is a viral sensor that detects whole range of RNA viruses, it presents a unique host target for broad-spectrum antiviral intervention. We have discovered that the anti-HBV compound SB 40 and its oral prodrug SB 44 also induce rapid translocation of RIG-I on dsRNA. Although HBV is a DNA virus, it uses a pregenomic RNA (pgRNA) template for the initiation of DNA synthesis; therefore RIG-I may be a receptor for HBV pgRNA as well. Hence, the mechanism of antiviral action of SB 40 and SB 44 may also include the induction of shuttling of RIG-I on pgRNA of HBV that inhibit viral replication. Based upon studies with SB 40 and SB 44, we have identified certain structural and stereochemical attributes of short oligonucleotides that are important for rapid RIG-I translocation on dsRNA and established a strong rationale for the design and synthesis of focused libraries for lead optimization and discovery of potent antiviral compounds.

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Efficacy of 2′-C-Methylcytidine Against Yellow Fever Virus in a Hamster Model of Disease

Justin Julander[1,*], Ashok Jha[2], Jung-Ae Choi[1], Don Smee[1], John Morrey[1], Chung Chu[2] [1] *Institute for Antiviral Research, Utah State University, Logan, USA* [2] *The University of Georgia, College of Pharmacy, Athens, USA*

Yellow fever virus (YFV) causes periodic outbreaks of acute disease despite the availability of an effective vaccine. The National Institute of Allergy and Infectious Disease (NIAID) has listed YFV as a Category C priority pathogen, thus prioritizing the development of therapeutic intervention strategies for the treatment of disease caused by this flavivirus. Derivatives of the nucleoside analog 2′-C-methylcytidine (2′-C-MeC) are effective in improving disease in people infected with hepatitis C virus, a related flavivirus, but gastrointestinal side effects have inhibited clinical development. The compound 2′-C-MeC was found to have activity against YFV in Vero cells, which was confirmed by a virus yield reduction assay. The 90% effective concentration (EC_{90}) in Vero cells was 0.32 μg/ml and the 50% cytotoxic concentration (CC_{50}) was 32 μg/ml, yielding an