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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68

Orally Bioavailable Anti-HBV Dinucleotide Acyloxyalkyl Prodrugs

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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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69

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

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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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70

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

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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

SI of 100. Time of addition studies demonstrated activity of this compound when added as late as 16 h after virus challenge of Vero cells with an EC_{90} of 8.9 μg/ml. Significant improvement in survival, serum levels of ALT, and virus titer in the liver was observed after bid treatment with 120 mg/kg/d of 2′-C-MeC for 7 days beginning just prior to virus challenge. A 4-day bid treatment regimen with this dose beginning 4 h prior to virus challenge was also effective in significantly improving survival. A lower dose of 80 mg/kg/d was also effective in significantly improving survival and serum ALT. Treatment with the 120 mg/kg/d dose initiated beginning 2 dpi was effective in significantly improving survival and serum ALT. Due to the severe gastrointestinal effects associated with long-term treatment, this compound and its derivatives may not be clinically viable for the treatment of chronic HCV. Alternatively, short-term treatment of an acute flaviviral disease like YFV would likely minimize or eliminate deleterious side effects associated with long-term treatment, potentially making the use of 2′-C-MeC and active derivatives a viable option for therapeutic intervention.

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71

Immunosafety Assessment of CD4 MAB-based Bifunctional HIV Entry Inhibitor (CD4-BFFI) using *In Vitro* Immunoassays

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We have previously described a CD4 monoclonal antibody (mAb)-based bifunctional HIV entry inhibitor (CD4-BFFI). CD4-BFFI demonstrated highly potent anti-HIV activities and excellent *in vivo* stability. Since CD4-BFFI binds to CD4 and CD4 is involved in $CD4^+$ T cell activation and other immunological functions, it is important to assess the potential immunological liabilities of CD4-BFFI before it enters human studies. We evaluated the direct effects of CD4-BFFI on $CD4^+$ T cells to see if it activates T cells via cross-linking CD4 molecules on cell surface. Our results showed that CD4-BFFI did not activate Jurkat cells or peripheral blood mononuclear cells (PBMC). There have been reports that some antibodies, especially those targeting blood cells, induced quick and marked cytokine release (cytokine storm) when dosed in humans, which may result in severe complications and even deaths. To assess the risk, an *in vitro* assay was performed using human whole blood from multiple donors. CD4-BFFI was incubated with human blood for 6 h, no cytokine secretion was observed, while the control anti-CD52 antibody (alemtuzumab) caused significant release of cytokine TNF-α and neutrophil activation (elevated CD11 expression) in 11 of the 12 donor blood samples. To investigate whether CD4-BFFI interferes with the co-receptor function of CD4 on T cells, an in vitro T cell activation assay was performed using Jurkat T cells and MACSiBeads that mimic antigen-presenting cells (APC). Significant activation of Jurkat cells was observed after stimulation with MACSiBeads. Co-incubation with CD4-BFFI showed no effects on Jurkat cell activation. Similar results were obtained using primary human PBMC cultures. An antigen-specific T cell activation assay was then developed for further evaluation. Human PBMC from cytomegalovirus (CMV)-infected donors was stimulated with CMV pp65 protein and significant activation of $CD4^+$ T cells (elevated CD69 and CD25 expression) was observed. Co-treatment with CD4-BFFI showed no effect on T cell activation. In summary, by using several in vitro immunoassays, we have demonstrated that CD4-BFFI did not activate human whole blood or T cells, and it did not interfere with the co-receptor function of CD4 in T lymphocytes in APC-mediated T cell activations.

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72

Inhibition of Severe Acute Respiratory Syndrome Coronavirus Replication in a Lethal SARS-Cov Balb/C Mouse Model by Stinging Nettle Lectin, Urtica Dioica Agglutinin (UDA)

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Keywords: BALB/c mice; SARS-CoV; Urtica dioica agglutinin (UDA).

Urtica dioica agglutinin (UDA) was tested for efficacy in a lethal SARS-CoV-infected BALB/c mouse model. UDA is a small plant monomeric lectin, 8.7 kDa in size, with an N-acetylglucosamine specificity and inhibits viruses from Nidovirales in vitro. In the current study, groups of BALB/c mice were infected with 2 LD50 of virus and treated intranasally with UDA at the doses of 20, 10, 5 and 0 mg/kg/day for 4 days beginning at 4 h post virus exposure. Treatment with UDA at 5 mg/kg significantly protected mice against a lethal infection with mouse-adapted SARS-CoV ($p < 0.001$), but did not significantly reduce virus lung titers. All mice receiving UDA treatments were also significantly protected against weight loss due to the infection ($p < 0.001$). UDA also effectively reduced lung pathology scores. All mice receiving poly IC:LC, the positive control drug, survived the infection ($p < 0.001$). At day 6 after virus exposure, all groups of mice receiving UDA or poly IC:LC had much lower lung weights than did the placebo-treated mice. Our data suggest that UDA treatment of SARS infection in mice leads to a substantial therapeutic effect that protects mice against death and weight loss.

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73

Viprolaxikine, a Novel Cytokine-like Protein from Insect Cell Cultures can Reduce Dengue-2 Virus Titres in Mammalian Cells

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Dengue virus (DEN) is an arthropod-born virus that causes dengue fever and dengue hemorrhagic fever in human hosts, but no disease in mosquito vectors. Viruses often persist in insects and other arthropods such as shrimp in either single, dual or multiple infections without gross signs of disease. From the supernatant solution of grossly normal C6/36 mosquito cell cultures persistently infected with DEN-2 virus, a novel antiviral agent was separated by ultrafiltration (5 kDa). Pre-incubation of mammalian (Vero) cell cultures with the ultrafiltrate reduced DEN-2 titres by up to 4 logs upon subsequent challenge. There was no reduction in titre for Vero cells pre-incubated with ultrafiltrate from uninfected C6/36 cells. Protease treatment of the protective ultrafiltrate removed its anti-DEN-2 activity while heating did not. Since 8-hr pre-incubation with the unltrafiltrate was required to obtain maximum protection against DEN-2, the active substance was called viprolaxikine,