strain A/VN/1203/2004 (H5N1) using a cell-based HTS assay. The screen yielded five active compounds (SI50 value > 3) representing two different classes of molecules, benzoquinazolinones and thiazoloimidazoles, which have not been previously identified as having anti-viral/anti-influenza activity. Subsequent synthetic work led to novel second-generation compounds with improved potency and solubility. Several compounds displayed significant antiviral activity with low EC_{50} values (nM range) without significant toxicity and high selectivity (SI50 > 3125) in MDCK cells. Time of addition experiments revealed that seven of these compounds inhibited an event early in the virus life cycle; suggesting they may affect entry. We screened our lead compound, SRI 22521, against 14 influenza A and B viruses in MDCK cells to establish its spectrum of activity. The compound was highly active against H1N1 and H5N1 viruses, but not active against H3N2 and B viruses. Preliminary neuraminidase assays reveal that SRI 22521 did not inhibit viral neuraminidase. Given the promising in vitro activity of SRI 22521, we examined its efficacy at 100 mg/kg of body weight/day (two times a day) for 5 days in mice challenged with $10LD_{50}$ of influenza virus A/VN/1203/2004. We noted a reduction in the rate of mortality in mice challenged with virus. Experimental results with this compound indicate that improving the solubility characteristics of this compound will be beneficial and work toward generating such compounds is in progress. We continue to explore the mechanism of action of this new and promising scaffold.

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Rimantadine and Oseltamivir Combination Effects in a Therapeutic Course of Application Against Influenza A (H3N2) in Mice

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Our previous studies demonstrated a marked synergistic combination effect of rimantadine and oseltamivir in 25:1 dose ratio in experimental infection with influenza A (H3N2) in mice when the treatment course begins on the day of virus inoculation (v.i.). Here we studied effect of both compounds in the same ratio and in optimal doses applied 24 h post v.i. in order to determine if the efficacy of combined therapy is preserved when the disease is already in progress which is the situation closer to the real conditions in patients. White male mice 16–18 g were inoculated intranasally with 0.05 ml/mouse of influenza A/Aichi/2/68 (H3N2) virus. Rimantadine hydrochloride and oseltamivir phosphate were administered per os twice daily in 5-day-treatment course beginning 24 h after v.i. with 10–20 MLD_{50}.Protection index (PI) and mean survival time (MST) were determined through 14 days post v.i. Combinations of 5, 10, 20, 40 and 80 mg/kg/day rimantadine and 0.2, 0.4, 0.8, 1.6 and 3.2 mg/kg/day oseltamivir were combined in doses ratio 25:1. The effects of 5 mg/kg oseltamivir + 40 mg/kg rimantadine and 10 mg/kg oseltamivir with 80 mg/kg rimantadine were studied, too. Combinations of 0.8 mg/kg oseltamivir + 20 mg/kg rimantadine, 1.6 mg/kg oseltamivir + 40 mg/kg rimantadine and 3.2 mg/kg oseltamivir + 80 mg/kg rimantadine demonstrated marked protective effects: PI values of 63.9%, 79.7% and 82.6% and MST of 12.8, 13.3 and 13.7 days, while the compounds' effects administered separately at the same doses vary from 15.7% to maximum 46.3% PI and 8.9 to 12.8 days MST. MST values in the placebo were 8.7 days. Combinations of 0.2 and 0.4 mg/kg oseltamivir with 5 and 10 mg/kg rimantadine, respectively, showed no protective effect. 5 mg oseltamivir + 40 mg rimantadine and 10 mg oseltamivir + 80 rimantadine combinations exert strong protective effect: 94.5% PI and 13.9 days MST and 100% PI and MST more than 14 days, respectively. Combination of oseltamivir and rimantadine in 1:25 dose ratio as well as in optimal in vivo doses administered in a therapeutic course(onset 24 h post virus inoculation) demonstrated a marked synergistic protective effect in mice experimentally infected with influenza virus A (H3N2).

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Combination of Neuraminidase Inhibitors with T-705 for Treating Influenza Virus Infections in Cell Culture and in Mice

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Effective treatment of influenza virus infections remains a public health priority. Use of combinations of antiviral compounds may increase efficacy and reduce the frequency of emergence of drug-resistant viruses. The viral neuraminidase inhibitors oseltamivir (an in vivo prodrug, or its active form oseltamivir carboxylate) and/or zanamivir were combined with T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide, currently in clinical trials) to treat infections in cell culture and in mice. MDCK cells were infected with influenza A/PR/8/34 (H1N1) and A/Victoria/3/75 (H3N2) viruses and treated with varying combinations of compounds. Additive to synergistic interactions was evaluated based upon reductions in viral cytopathology. These studies indicated several combinations of oseltamivir carboxylate or zanamivir with T-705 at low micromolar concentrations produced synergistic responses against both virus infections. Later, drug combination studies were conducted in BALB/c mice infected intranasally with influenza A/NWS/33 (H1N1), A/Victoria/3/75 (H3N2), and A/Duck/MN/1525/81 (H5N1) viruses using oseltamivir and T-705. Oral treatments were given twice a day for 5 (A/NWS infection) or 7 (A/Victoria and A/Duck infections) days starting 24 h after virus exposure. Synergistic activity was observed at the lower doses (20–25 mg/kg/day) of T-705, as determined by increases in numbers of survivors. Use of oseltamivir and T-705 in combination in the clinic may be warranted. [Supported in part by NIAID contracts NO1-AI-15435, NO1-AI-30048, and NO1-AI-30063.]

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High Throughput Screening of Protease Inhibitor Libraries Using a Novel Dual Pseudotype-Based Assay for SARS-CoV Entry

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The severe acute respiratory syndrome-associated coronavirus (SARS-CoV) recently emerged as the causal agent of an endemic atypical pneumonia, infecting thousands of people worldwide. We describe here a rapid and safe high-throughput assay for specifically screening for inhibitors of viral entry, using lentiviral pseudovirions whose entry is driven by SARS-CoV Spike glycoprotein. In preliminary studies, we found that many initial hits identified as potential inhibitors of entry mediated by SARS-CoV Spike, were also able to inhibit other pH-dependent viruses, likely due to gross effects on cellular function. In order to overcome this obstacle, in the same well, a second, unrelated pH-dependent viral envelope in conjunction with a lentiviral vector encoding a different reporter gene is

utilized in order to reduce the numbers of false positive hits. Both targeted and general small molecule libraries were screened for inhibitors of SARS-CoV entry, and a number of compounds were identified that inhibit SARS-CoV entry and replication. In particular, we took advantage of our previous findings that cathepsin L in target cells is required for activation of SARS-CoV Spike, in order to focus on libraries of cysteine protease inhibitors. 16 positive "hits" with 95% inhibition or higher in the primary screen were further studied for drug dose–response, cell toxicity, and the ability to inhibit coronavirus 229E, Ebola and live SARS-CoV. Three related compounds, exhibiting potent antiviral activity ($IC_{50} < 10^{-4}$ μM) were selected for small animal studies.

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Escaping Development of Drug-Resistant Mutants: Basis for Effective Chemotherapy of Enterovirus Infections

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Enteroviruses are causative agents of more than 50 various diseases, including meningitis, encephalitis, pleurodinia, myocarditis, pericarditis, insulin-dependent diabetes mellitus, etc. Most of enteroviruses induce several clinical syndromes, a phenomenon unique in the infectious pathology. The great majority of these infections are unapparent ones or have a subclinical course. Prevailing role in the strategy of counteraction of enteroviral infections is the use of anti-enteroviral chemotherapeutic agents administered during disease latency period—urgent prophylaxis. It might reduce to a minimum the risk of enterovirus induced myocarditis, acquired diabetes in infant age and other enterovirus infections with severe course. The main obstacle of the development of effective anti-enteroviral chemotherapy is the development of drug-resistance, phenomenon based on the unusually high level of mutation rate (10^{-3}). We carried out systematic study of the drug-resistance on the model of coxsackievirus B1 neuroinfection in mice treated with disoxaril, WIN compounds, binding to the hydrophobic pocket of enteroviral VP1 protein. In parallel, disoxaril-resistant and disoxaril-dependent Cox B1 mutants have been developed in vitro, in FL cells. Phenotypic characteristics, VP1 genome sequencing and VP1 protein sequence deduction of disoxaril mutants have been determined. Sequence changes gave satisfactory explanation for mutant resistance and on the unusual effect of inhibitor-dependence. Combination effects of anti-enteroviral agents with different modes of action have been carried out in cell culture experiments and a series of synergistic combinations have been selected. Administration of antivirals in synergistic combinations could be considered as a prospective approach to decrease the level of drug-resistance and to improve the chemotherapy efficacy. A new scheme of application of the partners in the synergistic combinations was developed on the model of experimental coxsackievirus B1 infection in newborn mice. The maximum protective effect was reached with the combination disoxaril/guanidine.HCl/oxoglaucine.

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Novel Small Molecule Inhibitors of Dengue Virus Replication

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There is an urgent need for new antivirals for both treatment and control of dengue virus, given that over 50 million people are infected worldwide every year and there are no approved vaccines or antiviral drugs available. An antiviral drug that inhibits viral replication without increasing the risk for antibody-dependent enhancement (ADE) of infection would be extremely valuable for public health by providing a means to control outbreaks, as well as to travelers to endemic regions. The goal of the SIGA dengue program is to develop a small molecule therapeutic for the treatment and/or prevention of disease caused by dengue virus, with a final drug product that will be a safe, effective, and orally administered antiviral compound. A sensitive and specific high throughput screening (HTS) assay has been developed to evaluate compounds from the SIGA chemical library for inhibitory activity against dengue-2 (DEN-2) virus replication. Hits have been identified that are potent (EC50 < 5 μM) and selective (CC50 > 50 μM), with initial structure activity relationship in several series of related compounds. Early hits have structures that are chemically tractable, in that they possess chemically stable functionalities and have potential drug-like qualities. Lead series have been identified with activity against all four serotypes of dengue virus which are being defined by spectrum of activity, mechanism of action, preliminary absorption, distribution, metabolism, and excretion (ADME) profiles, and pharmacokinetic (PK) evaluations. One of these series has shown proof-of-concept efficacy in a murine model of disease. The identification and characterization of early stage dengue virus inhibitors with activity in a murine model of dengue virus infection represents a compelling start toward our goal.

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Escaping Development of Drug-Resistant Mutants: Basis for Effective Chemotherapy of Enterovirus Infections

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Enteroviruses are causative agents of more than 50 various diseases, including meningitis, encephalitis, pleurodinia, myocarditis, pericarditis, insulin-dependent diabetes mellitus, etc. Most enteroviruses induce several clinical syndromes, a phenomenon unique in the infectious pathology. The great majority of these infections are unapparent ones or have a subclinical course. Prevailing role in the strategy of counteraction of enteroviral infections is the use of anti-enteroviral chemotherapeutic agents administered during disease latency period—urgent prophylaxis. It might reduce to a minimum the risk of enterovirus induced myocarditis, acquired diabetes in infant age and other enterovirus infections with severe course. The main obstacle for the development of effective anti-enteroviral chemotherapy is the development of drug-resistance, phenomenon based on the unusually high level of mutation rate (10^{-3}). We carried out a systematic study of the drug-resistance on the model of coxsackievirus B1 neuroinfection in mice treated with disoxaril, WIN compounds, binding to the hydrophobic pocket of enteroviral VP1 protein. In parallel, disoxaril-resistant