antiviral effect of a novel low-molecular polyvalent hemagglutinin inhibitor, containing Sia2-3Gal disaccharide motifs, on avian influenza A virus. *Methods*: Low-molecular polyvalent sialoside was studied as viral hemagglutinin inhibitor of avian influenza A (H5N1, H5N2, H5N3) virus strains in the inhibition assays of virus binding with fetuin molecules (FBI) and infectious focus forming in MDCK cells. To investigate the protective effect of hemagglutinin inhibitor in an animal model, we have infected mice with highly pathogenic influenza virus strain A/Chicken/Suzdalka/2005 (H5N1), isolated from poultry and wild birds in Western Siberia. To study the development of viral resistance to novel inhibitor we have conducted serial influenza virus passages in MDCK cells in the presence of increasing concentrations of hemagglutinin inhibitor. *Results*: The values of 50% inhibiting concentration (IC50) of hemagglutinin inhibitor obtained in MDCK cells and in FBI assay ranged from 1.5 to 10.0 μM for different influenza A virus strains. Intranasal administration of inhibitor (3.6 mg/kg) completely protected mice from infection of highly pathogenic avian strain A/Chicken/Suzdalka/2005 (H5N1). The results of passaging experiments indicated that hemagglutinin inhibitor showed no tendency to induce viral resistance. *Conclusion*: The data obtained in vitro and in vivo suggest that novel low-molecular polyvalent hemagglutinin inhibitor may be useful for the treatment of avian influenza virus infection.

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In Vitro Anti-Influenza Virus Effect of a Protease Inhibitor from a Streptomyces Strain

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Influenza viruses are important pathogens, causing infections both in humans and domestic animals. The virulence of these viruses depends on the ability of the hemagglutinin precursor (HA0) to be cleaved post translation to subunits HA1 and HA2 by trypsin-like proteases of the host. The inhibition of this cleavage by exogenous protease inhibitors may result in inhibition of subsequent rounds of viral replication. In the search for novel alternative approaches for the treatment of influenza infection we have studied the in vitro anti-influenza virus effect of a novel proteinaceous protease inhibitor (SS 34-1), isolated from the culture supernatants of a Streptomyces strain. The influenza virus-inhibitory effect was further studied with respect to the specificity and selectivity of viral inhibition. As a first approach we assessed the susceptibility of representative influenza viruses to the inhibitory action of SS 34-1; most sensitive to inhibition were A/Germany/34, strain Rostock (H7N1) and A/PR8/34 (H1N1). By the use of complementary virological assays it was demonstrated that the expression of the viral haemagglutinin on the surface of infected cells, the virus-induced cytopathic effect and the infectious virus yields, used as measures of A/Rostock virus growth, were all reduced at non-toxic concentrations of SS 34-1. In addition in preliminary experiments it the preparation protected mice from mortality in the experimental influenza A/Aichi/2/68 (H3N2) virus infection. All experiments were performed in parallel with the known proteolytic inhibitors ε-aminocaproic acid and aprotonin. The isolated novel protease inhibitor was purified by anion-exchange chromatography and reversed phase-HPLC analysis. It was a hydrophobic and a termostable protein, had a molecular mass of 11.2 kDa, isoelectric point of 7.5 and a high content of hydrophobic amino acids and proline. The N-terminal sequence demonstrated its homology to the *Streptomyces* subtilisin inhibitors family. The present results are in accordance with the findings that protease inhibitors of microbial origin could be used for the control of influenza virus infection.

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Protective Effect of a Fungal Superoxide Dismutase, Combined with a Plant Polyphenol Extract and Rimantadine Hydrochloride in the Murine Experimental Influenza Virus Infection

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The protective effect of a fungal Cu/Zn-containing superoxide dismutase, produced from *Humicula lutea* 103 (HL-SOD), applied in combination with a polyphenol extract, isolated from *Geranium sanguineum* L. (PC) or with rimantadine hydrochloride (Rim), was evaluated in the experimental influenza virus infection (EIVI) in mice, induced with virus A/Aichi/2/68 (H3N2). Preliminary results showed that HL-SOD caused neither acute nor chronic toxicity in the experimental animals, treated 4-fold with doses of 500 U/mouse/day. Rimantadine hydrochloride is an established selective antiinfluenza virus agent; in the dose 40 mg/kg, administered orally 24 and 2 h before and 24, 48 and 72 h after virus challenge, it protected mice from mortality (protective index, PI = 85.5%). The plant extract PC exhibited a pronounced antiinfluenza virus effect applied orally 3 h before viral infection in the dose 10 mg/kg (PI = 80.0%). HL-SOD, applied intravenously fourfold from 4 to 7 days after viral challenge in the dose 500 U/mouse/day also protected mice in the EIVI, PI = 86.1%). The intraperitoneal application of HL-SOD, a much more convenient way of treatment, was less effective. The combined application of HL-SOD, both intravenously and intraperitoneally, and PC or Rim in doses, which by themselves did not defend significantly mice, resulted in a synergistically increased protection, determined on the basis of protective indices and the amelioration of lung injury. Lung weights and consolidation as well as infectious lung virus titres were all decreased significantly parallel to the reduction of mortality rates; lung indices were raised. The excessive production of reactive oxygen species by alveolar macrophages as well as the elevated levels of the lung antioxidant enzymes superoxide dismutase and catalase, induced by EIVI, was brought to normal. For comparative reasons the combined protective effect of PC and the antioxidant vitamin C was investigated; synergistic enhancement of protection was observed. The results support the findings that the appropriate use of antiviral agents with alternative modes of action is a promising approach for the treatment of influenza virus infection.

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Discovery of New Inhibitors of the Influenza H5N1 Virus

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We screened the NIH Molecular Libraries Screening Centers Network (MLSCN) 100,000 compound library against influenza

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