HSV-1 following a single passage with the inhibitor in Vero cells. Furthermore, resistance selection occurred when the inhibitor was continuously present from prior to virus inoculation suggesting that certain resistance mutations may pre-exist in virus populations at relatively high frequency. PCR data will be presented to confirm these observations. It was shown subsequently that 2 out of 10 recent clinical isolates of HSV-1 also contained BAY 57-1293-resistant variants at 10^{-4} to 10^{-5} p.f.u. This is similar to the laboratory isolates and 10–100 times the previously reported spontaneous rate for HPI-resistance mutations (10^{-6}) in plaque-purified HSV-1 strains. The most common resistance mutations involved three amino acid residues just down-stream from the predicted helicase motif IV in HSV-1 UL5 and one residue near the C-terminus of the primase (UL52). We also showed that certain HPI-resistance mutations in UL5 are associated with increased or decreased virus growth in tissue culture with concomitant effects on pathogenicity.

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Murine Model of Recurrent Vaginal HSV-2 Shedding and Effect of Acyclovir

Rhonda Cardin*, Nicholas Farley, Fernando Bravo, Julie Earwood, Nancy Sawtell, David Bernstein

Cincinnati Children's Hosp, Med. Center, Cincinnati, USA

Genital herpes simplex type 2 (HSV-2) infection is common and leads to latency, reactivation, recurrent shedding and transmission. A murine model of recurrent genital HSV-2 shedding would be useful for characterization of the immune response to recurrent HSV-2 and for testing of novel antiviral therapies; however, genital HSV-2 infection of mice is lethal. In our studies, Swiss Webster mice were infected intravaginally with HSV-2 (strains 186 or MS) and treated with acyclovir (ACV) (100–150 mg/kg) beginning at 3 days post-infection (dpi) for 10 days. Animals became infected as evidenced by vaginal virus replication, developed local symptoms and survived (>80%) to 35 dpi. No replicating virus was detected in the dorsal root ganglia (DRG) after 30 dpi, yet all DRG samples were positive for HSV-2 DNA by PCR, indicating the establishment of latency. To determine whether recurrent HSV-2 vaginal shedding occurred, vaginal swabs were collected every 3 days beginning at 20 dpi and analyzed by PCR and plaque assay. Replicating virus was not detected in the vaginal samples, however, 47/59 mice (80%) shed virus as detected by nested PCR on at least 1 day, and virus was detected on approximately 15% of days. In another study, surviving ACV-treated mice were divided so that one group was again treated with ACV from 21–31 dpi and one group was not retreated. Retreated mice had increased survival, indicating that viral replication contributes to the eventual death of the mice. Mice receiving the second ACV treatment exhibited significantly less recurrent vaginal shedding during treatment (0.8% of swabs were HSV-2 positive) compared to 7.3% in mice not receiving ACV during this period ($P = 0.01$) supporting the validity of the recurrence model and its utility to evaluate antiviral therapies. Furthermore, initially ACV-treated mice receiving cyclophosphamide from d35–38 experienced increased mortality, supporting a role for immune control of the persistent infection. This model will be useful for evaluating anti-HSV therapies and will allow further evaluations of the immune mechanisms that control recurrent infections.

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Activation of Cyclopropavir Involves Unique Phosphorylation by Guanylate Kinase

Brian G. Gentry[1,*], Chengwei Li[2], John C. Drach[1], Jiri Zemlicka[2]

[1] *University of Michigan, Ann Arbor, USA;* [2] *Wayne State University, Detroit, USA*

Human cytomegalovirus (HCMV) is a widespread pathogen that can cause severe disease in immunologically immature and immunocompromised individuals. Cyclopropavir (CPV), a second generation methylenecyclopropane guanosine nucleoside analog, demonstrates in vitro activity against HCMV and MCMV with EC_{50}s of 0.27–0.49 mM [J. Med. Chem. 47: 566, 2004] and in vivo activity with a 2–5 log reduction in titers of virus in SCID mice (Antimicrob. Agents Chemother. 48: 4745, 2004). We have found that resistance of HCMV to CPV maps to a mutation in the viral UL97 gene resulting in a truncated UL97 protein devoid of both the ATP binding region and kinase activity domain (Antiviral Res. 78: A54, 2008). UL97 is the protein responsible for the initial phosphorylation of certain nucleoside analogs, all of which must be converted to a triphosphate to elicit antiviral activity. Consequently, it has been hypothesized that CPV must be converted to a triphosphate (CPV-TP) to give antiviral activity. Once CPV-MP is formed by UL97, we further hypothesize that guanylate (GMP) kinase is responsible for the conversion of CPV-MP to CPV-DP. Preliminary studies demonstrated that the S–(+)-enantiomer of CPV-MP was the preferred substrate for GMP kinase and was used for all subsequent experiments. Incubation of CPV-MP with bovine GMP kinase gave a linear increase of CPV-DP for over 30 min. Surprisingly, another compound consistent with CPV-TP was formed as well. We also observed a species variation in the formation CPV-TP. With bovine GMP kinase, formation of CPV-TP was the rate-limiting step whereas the conversion of CPV-MP to CPV-DP was rate limiting with porcine GMP kinase. There was a greater accumulation of CPV-TP with the porcine enzyme at 30 min (∼25% CPVTP, ∼5% CPVDP), while the opposite was true with the bovine enzyme (∼5% CPVTP, ∼70% CPVDP). In control experiments in which the enzyme was incubated with its natural substrate GMP, only GDP was formed. We conclude that unlike other antivirals such as acyclovir, cyclopropavir can be converted to its active triphosphate by a single cellular enzyme once the monophosphate is formed by a viral kinase.

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EBNA1—A virally encoded protein binds cellular host promoters in a unique sequence and directly interferes with cellular gene expression. Implications for genomics approaches in drug design

Allon Canaan*, Vincent Schulz, Milind Mahajan, Alexander Urban, Sherman Weissman

Yale University School of Medicine, New Haven, USA

Although most EBV carriers are healthy, several cancers were identified to be associated with EBV. One EBV protein of particular interest is EBNA1. It was found to be expressed in all forms of EBV infections and tumors and it is the only EBV protein being expressed in the common latency type I infection. It was demonstrated by others that specific inhibition of EBNA1's expression

resulted in tumor growth suppression. EBNA1 plays a key role in EBV's gene transcription and since EBV is maintained in the nucleus of the infected cell, EBNA1 has access to host genomic DNA, as well. Therefore, we examined whether it may bind to cellular sequences. Here we report testing this hypothesis in EBV-free cell lines which are engineered to express EBNA1. Initially, microarray analysis demonstrated changes in gene expression as a reflection of EBNA1's expression. Subsequently, an anti-EBNA1 antibody was used to produce chromatin immunoprecipitation DNA from these cell lines. These DNA samples were hybridized to human promoter arrays to reveal that host genomic sites had been complexed with EBNA1. These genomic binding sites consisted of coding and non-coding sequences from both DNA strands. We have managed to demonstrate the presence of the promoters for some of the genes depicted by the microarray expression analysis, and to confirm it by QR-PCR. Subsequently, we have identified an EBNA1 consensus sequence within the engaged cellular promoters. This sequence shows no similarity to the viral DNA binding sequence of EBNA1 or to any transcription factor. This dual transcription capacity improves the virus' ability to coexist within the infected cell by monitoring viral gene expression while actively and directly manipulating host gene expression. Our studies corroborate evidence of the crucial role EBNA1 plays in EBV etiology and neoplasm and suggest that a genomic approach is needed to monitor drug design directed to target EBNA1 as it is involved in various cellular gene expression changes. Currently, the lack of specific anti-EBV therapies generates adverse side effects for patients undergoing chemotherapy.

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Oral Session 5: Respiratory Viruses, Emerging Viruses and Biodefense

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Plenary: Filoviral Minigenome systems and iVLPs as tools for antiviral research

Stephan Becker

University of Marburg, Marburg, Germany

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Identification and Mechanistic Studies on a Novel Class of Influenza Virus Fusion Inhibitors

Evelien Vanderlinden[1,*], Nesrin Cesur[2], Zafer Cesur[2], Fusun Goktas[2], Mathy Froeyen[1], Charles Russell[3], Lieve Naesens[1]

[1] *Rega Institute for Medical Research, Leuven, Belgium;* [2] *Istanbul University, Faculty of Pharmacy, Istanbul, Turkey;* [3] *St. Jude Children's Research Hospital, Memphis, USA*

We here report on a new class of inhibitors of influenza virus hemagglutinin (HA)-mediated fusion, with a similar backbone structure as some reported fusion inhibitors, consisting of an aromatic cyclic system linked to a non-aromatic cyclic system via an amide bridge [Luo et al., Virology, 226:66–76 (1996)]. In Madin–Darby canine kidney (MDCK) cells infected with influenza virus A/H3N2 (X-31), the 50% effective concentration of the lead compound [4M] was 3.4 μM, as determined by microscopic examination of the viral cytopathic effect and MTS cell viability assay. The concentration producing 50% inhibition of cell proliferation was 89 μM. Similar activity was seen for other A/H3N2 strains, whereas no activity was noted for influenza A/H1N1 and B viruses. At 20 μM [4M], virus yield was reduced by 3 logs. In time-of-addition studies, [4M] lost activity when added 1 h or later post-infection, showing that [4M] inhibits an early step in virus replication. Definite proof for HA-mediated fusion as the antiviral target was provided by the inhibitory effect of [4M] on virus-induced red blood cell hemolysis at low pH. [4M]-resistant mutants, selected after three passages in MDCK cells in the presence of 20–150 μM [4M], were plaque-purified and sequenced. The two main amino acid substitutions associated with [4M] resistance were R220S and E57K, located in the HA1 and HA2 domain, respectively. This suggests that [4M] may bind to the same pocket of the influenza virus HA as the structurally unrelated H3-specific fusion inhibitor tert-butyl hydroquinone [Russell et al., PNAS, 105:17736–41 (2008)]. The mutants showed uncompromised fitness, with similar efficiency for binding and replication as wild-type virus. However, in the hemolysis assay, the mutants displayed an increased fusion pH. Molecular modelling of [4M] within the HA structure will help to design new [4M] derivatives with improved activity. Also, the activity against other virus subtypes (e.g. A/H5N1 and A/H7N7) is under investigation.

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Novel Broad-Spectrum Biopharmaceuticals: From HIV-1 to Pandemic Influenza A Virus

François Jean[1,*], Vesna Posarac[1], Peter Cheung[2], Martine Boutin[1], Heather Braybrook[1], Richard Harrigan[2]

[1] *Department of Microbiology & Immunology, University of British Columbia, Vancouver, Canada;* [2] *B.C. Centre for Excellence in HIV/AIDS, St. Paul's Hospital, Vancouver, Canada*

Recently, my laboratory has reported the identification of the most potent endogenous furin-directed inhibitor, Spn4A [K_i: 13 pM (Richer M. et al. PNAS 2004)]. Because of our previous success with the bio-engineered serpin α_1-PDX [K_i; 0.6 nM; (Jean F. et al. PNAS 1998)] as a protein-based therapeutic (Jean F. et al. PNAS 2000), we hypothesized that strategic manipulation of the furin-like cellular subtilase levels by Spn4A and Spn4A-engineered variants might provide a means of effectively inhibiting the subtilase-dependent proteolytic cleavage of viral envelope precursor glycoproteins in the host secretory pathway, a critical cellular event required for production of infectious progeny (e.g., HIV-1, highly pathogenic H5N1 influenza A virus, West Nile virus, Dengue virus).

In this study, we report our recent original work in the exciting field of protein-based inhibitors as broad-spectrum biopharmaceuticals. First, we describe the anti-proteolytic activities and anti-HIV properties of our novel recombinant adenovirus (Ad−) expressing Spn4A variants (Ad-Spn4A) in the host cell secretory pathway. We demonstrated that expression of Ad-Spn4A in MAGI-CCR5 cells completely inhibited the subtilase-dependent processing of the HIV-1 envelope precursor gp160 and resulted in a complete reduction of productive HIV-1 infection as determined by HIV-1 Tat-driven β-galactosidase activity and syncytia formation assays. Second, we demonstrated that our novel Ad-Spn4A variants also completely block the subtilase-mediated cleavage of the hemagglutinin H5 encoded by pandemic influenza A viruses (HK/97) and resulted in a complete block of syncytia formation in human A549 epithelial cells. The detailed cellular mechanism of action of our novel serpin-based antiviral strategy and the impact of our findings for developing a novel generation of broad-spectrum protein-based