ral activity was lost when MDM were cultured in low-adhesive conditions. HIV-1 infection in MDM has been shown to be influenced by integrin function, as seen by the antagonist-dependent inhibition of viral replication. Thus, our data supports the idea that blocking avb3 and avb5 integrins interaction with its ligand compromise HIV replication in MDM.

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Use of the HCV Cell Culture (HCVcc) System for Antiviral Drug Testing

Jodi Beattie*, Jacob Simmons, Amine Noueiry, Paul Olivo

Apath LLC, St. Louis, USA

Recently described HCV cell culture (HCVcc) systems have provided the opportunity to study the entire virus life cycle in vitro. We are developing protocols to maximize the usefulness of the HCVcc system for developing therapies, especially those directed against previously inaccessible steps in the viral replication cycle, such as entry, virion assembly and viral exit. Using a chimeric genotype 2a virus (J6/JFH-1/JC1), we have shown that virus titers remain relatively constant for at least 13 days post-electroporation in Huh7.5 cells even when media is harvested and replenished daily, making it possible to maximize yields. While developing virus yield assays, we found that after infection of Huh7.5 cells at low MOI, HCVcc titers were less than 3×10^2 TCID50/ml until d3 post-infection, resulting in a limited dynamic range for a virus yield assay. We therefore developed virus yield assays that focus on specific stages of the life cycle. In one protocol, we analyze intracellular HCV RNA levels in response to compound treatment after infection. In a second protocol, we analyze the effect of compound on the production of infectious particles after electroporation of infectious RNA. By design, both of these assays mimic treatment of acute infection. Therefore, we developed an assay that mimics chronic HCV infection. In this assay cells that are persistently producing high levels of infectious virus are treated with inhibitors to determine their effect on ongoing virus production. Our current efforts are focused on applying these acute and chronic models to an HCVcc system that expresses replication-dependent Renilla luciferase (Renilla J6/JFH-1/JC1). In initial studies we have found that cells infected with a low MOI of Renilla J6/JFH1/JC1 virus produce a robust Renilla chemiluminescence signal 48 h after infection. Ongoing efforts to further develop this system will provide simpler reporter-based virus yield assays that can be used to assess the effectiveness of antivirals that target virus entry, replication and egress.

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In Vitro Vascular Leak as a Model of Viral Hemorrhagic Fever

Rebecca Brocato*, Voss Thomas

Tulane School of Medicine, New Orleans, USA

Viral hemorrhagic fever (VHF) describes a group of diseases associated with infection by a number of genetically diverse, enveloped single-stranded RNA viruses including: (1) filoviruses, (2) arenaviruses, (3) flaviviruses, and (4) bunyaviruses. Although clinical presentations of VHF can vary by virus, a critical hallmark of human VHF infection is the loss of vascular barrier function resulting in changes in plasma volume and development of coagulation defects that can result in bleeding, pulmonary edema, and shock. Evidence suggests a role for innate and adaptive immune cells and mediators in the development of vascular leak in addition to direct infection of EC by virus. While the development of virus-specific antiviral therapies is critical to the treatment of VHF, development of therapeutics aimed at prevention of vascular leak may provide broad-spectrum treatment for a variety of infectious agents associated with VHF without a requirement for precise identification of the agent, often a challenge in VHF endemic regions of the developing world. Our laboratory has optimized an existing cell-based model of vascular leak that measures electrical resistance to allow screening of potential inhibitors of vascular leak in arenavirus-, bunyavirus-, and flavivirus-infected EC. Our results indicate that Pichinde' virus (arenavirus), Dengue virus (flavivirus), and Hantavirus (Bunyavirus) infection of EC induces a decrease in electrical resistance indicating and increase in vascular permeability that requires virus infection and/or stimulation with proinflammatory cytokines or chemokines. Using this model, a panel of small molecule inhibitors targeting with cell signaling pathways involved in EC structural or functional integrity have been screened with results supporting the hypothesis that host–cell targeting of EC may be useful in the treatment of VHF. Taken together, these data support the use of this assay as a screen for active compounds for viral cellular targets associated with VHF while also identifying additional therapeutic targets for drug discovery.

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Resistance to Pyrimidinedione HIV Inhibitors Requires Multiple Mutations in Reverse Transcriptase, Envelope and Core Proteins

Robert W. Buckheit Jr.*, Christa E. Buckheit, Robert W. Buckheit III, Karen M. Watson

ImQuest BioSciences, Inc., Frederick, USA

The pyrimidinediones are small molecule HIV inhibitors with two distinct mechanisms of action, inhibiting HIV-1 RT at subnanomolar concentrations through interaction at the hydrophobic NNRTI binding pocket and the entry of both HIV-1 and HIV-2 at low nanomolar concentrations by interaction

with a novel conformational structure formed upon coculture of virus and cells. The pyrimidinediones inhibit the replication and transmission of resistant viruses bearing RT or Env mutations alone (albeit with 100-fold loss of sensitivity to NNRTI-resistant viruses) and remain highly active against multi-drug resistant (MDR) viruses with mutations in RT and/or protease. With serial passage in increasing compound concentrations, a virus which is completely resistant to the selecting pyrimidinedione can be selected. The selection follows a defined progression consisting of the initial appearance of mutations in the RT, resulting in approximately 100-fold loss in sensitivity, followed by the accumulation of mutations in gp120, gp41 and gag proteins which allow the virus to escape entry or maturation inhibition, yielding viruses with 1000–10,000-fold resistance. Finally, multiple additional changes occur in RT, resulting in complete resistance. The mutational profile suggests that the compounds act as typical NNRTIs but target a unique conformational structure to inhibit entry requiring interaction of envelope and gag proteins. Each of the resistant viruses have been evaluated for their sensitivity to other nonnucleoside, nucleoside and nucleotide RT inhibitors, entry and fusion inhibitors, and protease inhibitors. Cross-resistance is only detected with other NNRTIs. In light of the ability of the pyrimidinediones to inhibit entry and cell–cell fusion, it is notable that the viruses do not have cross-resistance to Fuzeon. The pyrimidinediones thus represent excellent therapeutic and microbicide development candidates based on the inability of viruses resistant to one of the mechanisms of action to abrogate the activity of the second mechanism. The pyrimidinediones have the potential to replace Sustiva and Fuzeon in current therapy regimens, but with one small molecule with a higher genetic barrier to resistance.

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Azaindole-based HIV-1 Integrase Specific Inhibitors Display Potent Anti-Retroviral Activity

Scott Butler[1,*], Michael Plewe[2], Atsuo Kuki[2], Teddy Johnson[2], Qinghai Peng[2], Dorothy Delisle[2], Joanne Graham[2], Hua Wu[2], Amy Patick[2]

[1] Pfizer Global R&D, Sandwich Labs, Sandwich, United Kingdom; [2] Pfizer Global R&D, La Jolla Labs, La Jolla, USA

We have synthesized and studied a series of compounds that share an azaindole core and additional groups such that the expected conformation would form a two-metal binding motif. We the characterization of the representative compound, PF-00558475, demonstrating that it is a potent and selective inhibitor of the strand transfer activity of HIV-1 Integrase and displays corresponding viral activity in cell culture HIV infections. We present biochemical, antiviral, and mechanistic studies indicating specific inhibitory activity against the HIV-1 Integrase enzyme. In addition, we have carried out resistance selection studies confirming that mutations to the Integrase gene are necessary and sufficient to reduce HIV-1 susceptibility to PF-00558475. Finally, we present results from a panel of clinical isolates indicating that this compound is a broad spectrum inhibitor of HIV-1.

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Establishment of a Cell-based HTS System for Discovery of Anti-Flavivirus Drugs

Yali Chen*, Chris Harver, Travis Warren, Dennis Hruby, Robert Jordan

SIGA Technologies, Corvallis, USA

Flavivirus infections have become a global public health concern due to the severe nature of disease caused by these viruses and their prevalence in the human population. There is an urgent need for specific antiviral therapies to treat these infections. The viral NS3 serine protease is an attractive target for anti-flavivirus therapy because it is highly conserved and essential for viral replication. We have designed a cell-based high throughput screening system for the identification of small molecule inhibitors of the flavivirus protease. In this system, two expression constructs were generated that constitutively express a bi-cistronic mRNA encoding the viral protease; or a proteolytically inactive mutant protease, and a marker gene (GFP^{CSI}) containing a specific cleavage-site for the viral protease inserted within the GFP. Cleavage at this site by the viral protease results in a loss of GFP fluorescence. Coexpression of NS3 protease of Dengue Virus type 2 with the engineered GFP^{CSI} in 293T cells results in site-specific cleavage of GFPCSI, destabilization of GFPCSI conformation, and reduced fluorescence. The relative fluorescent signal for GFPCSI in cells expressing wild-type NS3 (2.3 MFI) was reduced to $72 \pm 5\%$ of that in cells expressing the active site mutant $NS3^{S135A}$ (3.2 MFI) as measured by flow cytometry. The system has been validated by quantifying the level of inhibition of proteolytic activity in the presence of known protease inhibitors and experimental compounds. In the presence of Aprotinin, a known serine protease inhibitor, the proteolytic inhibition was 67%. Likewise, an inhibitor compound (ST905) identified by high throughput screening that reduced NS3-specific protease activity by 51% at 5 μM showed an 18% inhibition of GFP cleavage in the cell-based assay. Taken together, the cell-based HTS system will facilitate discovery of anti-flavivirus therapeutics.

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Mevastatin Markedly Potentiates the Anti-HCV Activity of Selective Inhibitors of HCV Replication and Delays or Prevents the Emergence of Antiviral Resistance

Leen Delang*, Johan Neyts

Rega Institute for Medical Research, K.U. Leuven, B-3000 Leuven, Belgium

Statinsare 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors that are widely used for the treatment of hypercholesterolemia. Recently, it was reported that certain