survival rates. The other monotherapy and double combination regimens provided no protection or only partial protection (0–60% survival) in each study. A comparison of the Kaplan–Meier survival curves showed that TCAD provided significantly better protection ($P < 0.05$) than all other regimens in both studies. TCAD produced survival rates in each study that were greater than the additive rates of each drug as a monotherapy, indicative of synergy. Moreover, TCAD reduced the magnitude of weight loss in infected animals significantly relative to all other treatments. Importantly, AMT contributed to the efficacy of TCAD against the AMT-resistant novel A/H1N1 virus. The TCAD regimen is highly active in two lethal mouse influenza treatment models against susceptible and resistant viruses. These results validate and build upon the previously demonstrated superior *in vitro* efficacy of TCAD versus monotherapy and double combination regimens and translate them into an *in vivo* model.

doi:10.1016/j.antiviral.2010.02.350

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In Vitro and *In Vivo* Efficacy of Combinational Therapy with Favipiravir (T-705) and Oseltamivir Against Influenza A/CA/04/09 Pandemic H1N1 Virus

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Serious disease caused by emerging pandemic influenza A (H1N1) viruses and the possibility of drug resistance underscores the need for different approaches for treating influenza infections. The augmentation of current monotherapies with newly developed agents targeting alternative functions of the influenza virus replication cycle may be a solution. The current studies demonstrate the efficacy of using a combination of two drugs with different mechanisms of action to enhance total anti-H1N1 influenza A activity compared to each compound alone. Combinations of oseltamivir, a currently used, clinically approved neuraminidase inhibitor and favipiravir, an experimental viral RNA polymerase inhibitor, were evaluated alone and in combination for *in vitro*/*in vivo* efficacy against a pandemic H1N1 influenza A virus. *In vitro* combination studies revealed synergy with 0.032–1.0 μM oseltamivir combined with 0.32–10 μM T-705. In an H1N1 lethal mouse model it was found that: (1) orally administered combinations of favipiravir at 30–0.3 mg/kg/day and oseltamivir at 3 mg/kg/day resulted in significant protection against death ($P < 0.001$) and in total survivors ($P < 0.05$–0.01), (2) the combinations of favipiravir and oseltamivir at higher doses ameliorated the weight loss attributable to virus infection, (3) the combinations of favipiravir and oseltamivir at higher doses were highly synergistic, and (4) the use of favipiravir at 30 mg/kg/day or higher may permit the use of lower doses of oseltamivir to achieve efficacy against pandemic H1N1 viruses. The results suggest that these two compounds could be used in combination to treat serious infections in humans caused by pandemic H1N1 viruses.

Acknowledgement: This work was supported by Contracts NOI-AI-30048 and NOI-AI-30063 (awarded to Southern Research Institute) from the Virology Branch, DMID, NIAID, NIH.

doi:10.1016/j.antiviral.2010.02.351

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Antiviral Activity of Leflunomide Against Respiratory Syncytial Virus

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Respiratory syncytial virus (RSV) is a major cause of often serious, fatal respiratory disease in infants and young children, organ transplant recipients, patients suffering cystic fibrosis or congenital heart disease, and the elderly. Severe RSV disease is characterized by a disproportionately intense pulmonary inflammatory response resulting in bronchiolar injury and compromise of airway function. Current treatment options are limited to ribavirin, which must be administered by small-particle aerosol for 12–18 h per day for 3–7 days, and passive immunoprophylaxis with monoclonal antibody specific for the RSV fusion protein (palivizumab), neither of which has been shown to reduce mortality. Leflunomide is an orally bioavailable anti-inflammatory drug approved for treatment of rheumatoid arthritis and currently in clinical trials as an immunosuppressant in transplant recipients. We have previously demonstrated that leflunomide exerts potent antiviral activity against CMV, HSV, and polyomavirus BK. We now report on the antiviral activity of this agent against RSV. Phase contrast microscopy and immunohistochemical staining demonstrated nearly complete attenuation of RSV-induced syncytia formation in infected human airway epithelial cell cultures treated with A77 1726, the active metabolite of leflunomide. Plaque assay of virus yield in RSV-inoculated cultures demonstrated potent, dose-dependent A77-mediated reduction in virus production. Likewise, pulmonary viral loads in RSV-inoculated cotton rats were reduced by >3 logs by leflunomide compared with vehicle-treated controls, even when leflunomide treatment was delayed until day 3 post-inoculation. Real-time rt-PCR demonstrated A77-mediated inhibition of viral genomic RNA synthesis and inhibition of transcription of several viral genes. Data generated by these experiments implicate leflunomide as a unique bifunctional agent with potential to both reduce viral load and, by virtue of its well-documented anti-inflammatory activity, attenuate the destructive inflammation associated with RSV disease. Sidwell and Barnard have stated that effective therapeutic intervention for severe RSV disease must include both antiviral and anti-inflammatory components. Leflunomide, it seems, effectively meets these criteria.

doi:10.1016/j.antiviral.2010.02.352

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Small-molecule Inhibition of Respiratory Syncytial Virus Fusion: *It Takes Two to Tango*

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Six-helix bundle (6HB) formation is an essential step for many viruses that rely on a class I fusion protein to enter a target cell and initiate replication. Because the binding modes of small molecule inhibitors of 6HB formation are largely unknown, precisely how they disrupt 6HB formation remains unclear, and structure-based design of improved inhibitors has thus been very speculative. It is currently believed that such inhibitors completely prevent 6HB formation by binding in a hydrophobic pocket composed of amino

acids from the HR1 central trimeric coiled-coil of the 6HB. To obtain more information on the precise binding mode and mechanism of inhibition of the potent RSV inhibitor TMC353121 (Bonfanti and Roymans, 2009), we determined the high resolution crystal structure of the compound bound at a hydrophobic pocket of the 6HB (Roymans et al., 2009). In contrast to what is generally believed, the binding site of TMC353121 is formed by amino acids from both HR1 and HR2. Binding of TMC353121 stabilizes the interaction of HR1 and HR2 in an alternate conformation of the 6HB, in which direct binding interactions are formed between TMC353121 and both HR1 and HR2. Rather than completely preventing 6HB formation, our data indicate that TMC353121 inhibits fusion by causing a local disturbance of the natural 6HB conformation. If binding with both HR1 and HR2 is a general requirement for the inhibition of 6HB formation by small-molecules, these results may fuel the structure-based discovery of other fusion inhibitors targeting viruses that use class 1 fusion proteins.

References

Bonfanti, J.F., Roymans, D., 2009. Prospects for the development of fusion inhibitors to treat human respiratory syncytial virus infection. Curr. Opin. Drug Discov. Dev. 12, 479–487.

Roymans, D., De Bondt, H.L., Arnoult, E., Geluykens, P., Gevers, T., Van Ginderen, M., Verheyen, N., Kim, H., Willebrords, R., Bonfanti, J.F., Bruinzeel, W., Cummings, M.D., van Vlijmen, H., Andries, K., 2009. Binding of a potent small-molecule inhibitor of six-helix bundle formation requires interactions with both heptad-repeats of the RSV fusion protein. Proc. Natl. Acad. Sci. U.S.A. (December) [Epub ahead of print].

doi:10.1016/j.antiviral.2010.02.353

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Enterovirus 3C Proteases: Structure-based Discovery of Inhibitors

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Crystal structures have been determined for the 3C proteases of coxsackievirus B3 and enteroviruses 68 and 71 in our laboratory, and for poliovirus, rhinovirus, and hepatitis A virus in other institutions. We are using these structural data for virtual screening and for fragment-based design of non-peptidic inhibitors. Fragment-screening using saturation-difference-transfer (STD) NMR spectroscopy turns out to be particularly successful in identifying small molecules (<300 kDa) that bind to the target. These binding events are confirmed by surface plasmon resonance and X-ray crystallography. Fragments are subsequently linked by medicinal chemistry. An interesting approach to self-ligation of fragments has been developed. An overview of 3C(pro) inhibition, with the latest results included, will be provided.

doi:10.1016/j.antiviral.2010.02.354

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A Novel 9-Arylpurine Acts as a Selective Inhibitor of *In Vitro* Enterovirus Replication Possibly by Targeting Virus Encapsidation

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We identified a class of 9-arylpurines as selective inhibitors of the replication of various enteroviruses; analogue TP219 [9-(3-acetylphenyl)-6-chloropurine], was selected for further studies. The antiviral activity was assessed by means of CPE and virus yield reduction assays, q-RT-PCR, bioluminescence and antigen detection. TP219 did not inhibit early steps (entry/uptake) nor did it affect polyprotein synthesis/processing or the synthesis of viral RNA, thus suggesting that the drug interacts at a late stage in the replication cycle. Drug-resistant variants were selected that are not cross-resistant to other classes of enterovirus inhibitors (including 3A, 2C and a 3D inhibitor); and they were found to carry several mutations in VP1 and VP3. These mutations were reintroduced in the wild-type genome to confirm their role in the drug-resistant phenotype. The above described experiments revealed that TP219 probably prevents the correct encapsidation of the virion. Mammalian two-hybrid studies are used to explore whether TP219 hinders VP1/VP3 interactions. To study whether the drug prevents virion assembly, we optimized and implemented the nanoLC–MS/MS based SILAC-assays. This allows quantification of capsids and precursors in the infected cells. Since (inhibition of) assembly as such is not a critical step in virus induced cell lysis, probably other (cellular) mechanisms are involved. To study whether specific conformational changes in the capsid inhibit a particular 'death signal' eventually leading to inhibition of virus induced cell death, the effect of TP219 on different apoptosis pathways (in infected and uninfected cell cultures) is being studied. Together, these studies may provide exciting insights in an entirely novel strategy to inhibit picornavirus replication.

doi:10.1016/j.antiviral.2010.02.355

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Is the Large T Antigen a Target for the Inhibition of SV40 Replication?

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Background: Simian virus (SV40) belongs to the polyomaviruses, a small DNA virus family that causes severe diseases in immunocompromised patients. The large T antigen (LTag) encoded by SV40 is involved in viral replication and in transformation. The in vitro activity of acyclic nucleoside phosphonates against SV40, especially cidofovir (CDV, HPMPC, Vistide®), has already been shown. But the mechanism of action has not been elucidated yet. Polyomaviruses do not express their own DNA polymerase and require the cellular DNA replication machinery for replication of their DNA.

Methods: CDV resistant SV40 clones were selected for their ability to grow in presence of the drug and their genome sequenced to identify mutations in the LTag gene. In addition, phenotyping of