References

Enders, D., Iffland, D., 2007. Synthesis, 1837–1840 (references therein).
Braverman, S., Pechenick-Azizi, T., Major, D.T., Sprecher, M., 2007. J. Org. Chem. 72, 6824–6831 (references therein).
de Castro, S., Lozano, A., Peromingo, M.T., Camarasa, M.J., Velázquez, S., 2008. Chem.-A: Eur. J. 14, 9620–9632.

doi:10.1016/j.antiviral.2009.02.042

38

A Small Llama Antibody Fragment Efficiently Inhibits the HIV Rev Multimerization *In Vitro*

Thomas Vercruysse[1,*], Els Pardon[2], Jan Steyaert[2], Dirk Daelemans[1]

[1] *Rega Institute for Medical Research, Katholieke Universiteit Leuven, B-3000 Leuven, Belgium;* [2] *Structural Biology Brussels, Vrije Universiteit Brussels, and Department of Molecular Interactions, VIB, B-1050 Brussels, Belgium*

The human immunodeficiency virus type 1 Rev protein is essential for the expression of single spliced and unspliced HIV mRNA, encoding for the structural proteins of the virus. In order to do so, Rev binds as a multimeric complex to the Rev responsive element (RRE)-containing mRNA and transports it from the nucleus to the cytoplasm exploiting the CRM1-mediated cellular machinery. An important aspect of the Rev function is its requirement for multimerization. We have used a unique strategy to identify a multimerization inhibitor of Rev based on the isolation of llama single-domain antibodies. The *Camelidae*, besides containing conventional antibodies consisting of heterodimers of a heavy and a light-chain, also contain heavy-chain antibodies that are homodimers of heavy-chain only. Therefore, single-domain antigen-binding fragments (VHHs) can be easily generated from the variable domain of these heavy-chain antibodies. These VHHs, also called nanobodies, are minimally sized, highly soluble entities that bind the antigen with nanomolar affinity. Our strategy consisted of producing single-domain nanobodies against HIV Rev by immunizing a llama with recombinant Rev protein. Using a FRET-based multimerization assay we discovered a nanobody that efficiently inhibits the Rev oligomerization *in vitro*. Our results suggest that the oligomeric assembly of Rev may represent a new approach to the chemotherapy of HIV.

doi:10.1016/j.antiviral.2009.02.043

39

Study of the Retention of Metabolites of 4′-ED4T, A Novel Anti-HIV-1 Thymidine Analog, in Cells

Xin Wang[1,*], Hiromichi Tanaka[2], Masanori Baba[3], Yung-Chi Cheng[1]

[1] *Department of Pharmacology, Yale University School of Medicine, New Haven, USA;* [2] *School of Pharmaceutical Sciences, Showa University, Tokyo, Japan;* [3] *Division of Antiviral Chemotherapy, Center for Chronic Viral Diseases, Graduate School of Medical and Dental Sciences, Kagoshima University, Kagoshima, Japan*

2′,3′-Didehydro-3′-deoxy-4′-ethynylthymidine (4′-Ed4T), a novel thymidine analog, has potent anti-human immunodeficiency virus type 1 (HIV-1) activity than its progenitor stavudine (D4T). The profile of intracellular 4′-Ed4T metabolites was qualitatively similar to that of zidovudine (AZT) but not to that of D4T, while it showed more persistent anti-HIV activity after drug removal than AZT or D4T in cell culture. When the CEM T cells were exposed to higher concentrations (2, 5 and 10 μM) of 4′-Ed4T, the amounts of major metabolite 4′-Ed4TMP increased proportionately. Furthermore, the higher amount of intracellular metabolites, especially 4′-Ed4TMP, brought about much longer retention of 4′-Ed4TTP after drug removal. We further investigated the efflux profiles of 4′-Ed4T in the comparison with AZT in CEM cells. After drug removal, both 4′-Ed4T and AZT were efflux from the cells in a time and temperature-dependent fashion. 4′-Ed4T was efflux from cells in its nucleoside form, while AZT was efflux from cells in its nucleoside and monophosphate (MP) form. The efflux of 4′-Ed4T from cells was much less efficient than that of AZT and kept higher amount of intracellular 4′-Ed4TMP than AZTMP. Dipyridamole could inhibit the efflux of AZT but not 4′-Ed4T in a dose dependent manner. The mechanism study showed that dipyridamole-dependent efflux of AZT nucleoside might due to an unknown transporter which was not related to the equilibrative nucleoside transporters. The effect of dipyridamole on AZTMP efflux might come from the inhibition of multidrug resistance protein 4 (MRP4). Those results demonstrated that less efficient efflux of 4′-Ed4T might be one of the biochemical determinants for its persistent antiviral activity in the cell culture.

Acknowledgments: Supported by AI-38204 from NIAID, NIH.

doi:10.1016/j.antiviral.2009.02.044

40

A High-content Screening Approach to Identify Compounds that Interfere with the Formation of the Hepatitis C Virus Replication Complex

J.M. Berke*, D. Fenistein, F. Pauwels, O. Lenz, T.-I. Lin, E. Krausz, G. Fanning

Tibotec BVBA, Generaal de Wittelaan L11B 3, 2800 Mechelen, Belgium

The hepatitis C virus (HCV) subgenomic replicon is routinely used in large screening campaigns to identify compounds that inhibit HCV RNA replication. Commonly used subgenomic replicons contain the HCV non-structural proteins NS3, NS4A, NS4B, NS5A and NS5B (NS3–NS5B) that assemble into membrane-associated replication complexes. These complexes are represented by "dot-like" structures when standard fluorescence microscopy techniques are applied. To screen compound libraries for inhibitors that interfere with formation of the HCV replication complex independent of replicon replication and cell cycle, we developed a high-content based assay utilizing inducible expression of the HCV non-structural proteins. A stable cell line was generated in which a fluorescent protein (GFP) was fused to NS5A for detection of replication complexes in fluorescence microscopy. HCV polyprotein expression was well regulated with doxycycline, and polyprotein processing appeared unaffected by the GFP insertion within NS5A. The morphology of the replication complexes was "dot-like" in appearance and comparable to what has been observed in replicon cells. Draq5 staining of nuclei and cytoplasm prior to assay readout allowed development of image analysis tools that simultaneously detected nuclei, cytoplasm and replication complexes as well as parameters that could indicate compound toxicity. As expected, replication complex formation was not affected in the presence of a polymerase inhibitor, whereas incubation in the presence of a protease inhibitor induced a dose dependent reduction of "dot-like" structures. Image analysis of the effect of a selection of replicon hits and kinase inhibitors on replication complex formation in this assay is currently ongoing. In conclusion, we have designed a high-content based assay to identify and characterize compounds that

interfere with the formation of the HCV replication complex. This approach represents a valuable tool for screening of HCV inhibitors with a novel mode of action as well as for the characterization of replicon hits.

doi:10.1016/j.antiviral.2009.02.045

41

Novel HCV Replication Mouse Model Using Human Hepatocellular Carcinoma Xenografts

Carl Guévin*, Alain Lamarre, Patrick Labonté

INRS-Institut Armand-Frappier, Laval, Canada

Background: Hepatitis C virus (HCV) infection causes chronic liver diseases and is a global public health problem. The only established animal model of HCV infection is the chimpanzee. In view of the limited availability of these animals, expense and ethical aspects, the establishment of a small-animal model for the study of HCV infection is a high priority. In absence of an immunocompetent mouse model for HCV replication, we developed a convenient xenograft mouse model that produces infectious viral particles.

Methods: To produce xenograft human hepatocellular carcinoma (HCC), we developed a highly tumorigenic Huh7 cells population able to promote the formation of HCC subcutaneously in SCID/beige mice. Huh7-7 cells are permissive cell line for cell culture of HCV particles (HCVcc) and were obtained through successive *in vivo* passages of tumor cells performed by subcutaneous transplantation of tumor fragments from the previous *in vivo* passage.

Results: Following injection with HCV-infected Huh7-7 cells, HCV RNA rose in the mouse sera and plateau at 10^3–10^5 GE/ml. Quantitative RT-PCR showed that up to 10^7 GE/μg of total RNA are present within tumors. Furthermore, a direct correlation between the size of tumors and the level of HCV RNA in the tumor was observed. Immunohistochemistry analysis of infected tumor tissue showed that the virus is widely spread within the tumor. Moreover, virus recovered from infected mice is infectious in cell culture. Finally, we showed that interferon-α and the protease inhibitors BILN-2061 both inhibited HCVcc strain JFH1 replication *in vivo*.

Conclusions: Human trials are realized solely based on efficacy data collected *in vitro* and on safety and pharmacokinetic profiles. The simplicity and the convenience of the model present here, should allow its utilization at an early stage in the compound profiling and give a more accurate indication of the compounds ability to block viral replication and infection in an *in vivo* setting.

doi:10.1016/j.antiviral.2009.02.046

42

In Vitro Activity and In Vivo Pharmacokinetics of Highly Potent Phosphoramidate Nucleoside Analogue Inhibitors of Hepatitis C NS5B

J. Hutchins[1,*], S. Chanberlain[1], C. Chang[1], B. Ganguly[1], E. Gorovits[1], A. Hall[1], G. Henson[1], A. Kolykhalov[1], Y. Liu[1], J. Muhammad[1], P. Perrone[2], A. Gilles[2], S. Holl[2], K. Madela[2], C. McGuigan[2], J. Patti[1]

[1] *Inhibitex, Alpharetta, USA;* [2] *Cardiff University, Cardiff, Wales, United Kingdom*

Phosphoramidate nucleoside analogues, or pronucleotides (ProTides), possess a number of pharmacological advantages over their parent nucleoside including a significant increase in antiviral activity, higher concentrations of triphosphate in liver, and potentially less toxicity due to reduced systemic nucleoside exposure. The in vitro and in vivo properties of a series of aminoacyl ProTides of 2′-C-methyl guanosine (2′-C-MeG) have been characterized. ProTides exhibited anti-HCV replicon activity as much as 20-fold greater than the parent nucleoside, with EC90's ranging from 200 to 800 nM. The compounds were synergistic when combined with ribavirin or interferon-2α. Cytotoxicity was not observed in Huh-7 cells (CC50 > 100 μM). In the more sensitive MT-4 cell line, CC50 values ranged from 20 to >100 μM. Culturing CEM cells with ProTides for 3 days at 100 μm or 13 days at 5 μM had no significant effect on mitochondria copy number. In primary human hepatocytes, conversion of ProTides to the triphosphate was measured, with C_{max} = 78 pmol/10E6 cells which is approximately 20-fold greater than the IC90. Therefore, ProTides of 2′-C-MeG exhibited excellent therapeutic indices and conversion to 2′-C-MeGTP in primary human hepatocytes exceeding the IC90. In PK experiments designed to measure plasma concentrations of the ProTides and parent nucleoside in the peripheral circulation and portal vein of cannulated cynomologus monkeys, efficient extraction by the liver was observed as indicated by low systemic levels of the ProTides. Triphosphate levels exceeding the IC90 were measured in primate liver biopsies following oral dosing. The primate PK study data indicate delivery of the ProTides to the liver and subsequent conversion to the triphosphate after oral administration. The findings of these studies support the continued development of 2′-C-MeG ProTides for the treatment of HCV infections.

doi:10.1016/j.antiviral.2009.02.047

43

Hepatitis C Virus NS5A Protein In Vitro Modulates Template Selection by the RNA-dependent RNA Polymerase

Olga Ivanova[1,*], Vera Tunitskaya[1], Alexander Ivanov[1,2], Vladimir Mitkevich[1,2], Vladimir Prassolov[1], Alexander Makarov[1], Marina Kukhanova[1], Sergey Kochetkov[1]

[1] *Engelhardt Institute of Molecular Biology, Moscow, Russia;* [2] *University of Oslo, Center for Medical Studies in Russia, Moscow, Russia*

Hepatitis C virus (HCV) infection is one of the most dangerous human diseases. The HCV replication complex is composed of viral nonstructural proteins including NS5B (RNA-dependent RNA polymerase, RdRp) and NS5A and of several cellular proteins. Since the recombinant NS5A protein can directly interact with NS5B and with viral RNA, it was proposed that NS5A plays an important role in virus replication.

NS5A is presented in infected cells in an unphosphorylated and two phosphorylated forms (basal and hyperphosphorylated). Basal NS5A phosphorylation occurs in the C-terminus and is catalyzed by casein kinase (CK) II, whereas hyperphosphorylation is accounted for by CKI. Although basal NS5A phosphorylation has no effect on HCV replication, its effect on the protein interaction with HCV RdRp and with RNA is unknown.

Here we demonstrate that unphosphorylated NS5A protein inhibits HCV RdRp activity in vitro in an artificial polyA-oligoU system but has only minor inhibitory activity on synthesis of viral RNA. In contrast, the phosphorylated CKII NS5A protein does not block polyA-dependent polyU synthesis but completely abolishes viral (−)-3′UTR replication and significantly inhibits (+)-3′UTR synthesis. The NS5A phosphorylation with CKI does not change the RdRp activity in any system. Phosphorylation of NS5A with CKII has no effect on the protein affinity to RdRp or RNA. By UV-crosslinking and RNA filter-binding experiments we revealed that NS5A prevented binding of the template to the polymerase. The presented mecha-