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

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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.

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

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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.

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

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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