The Koala and its Retroviruses:
Implications for Sustainability and Survival

edited by

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Technical Reports of the Australian Museum, Online (2014) No. 24, pp. 65–69.
ISSN 1835-4211 (online)
http://dx.doi.org/10.3853/j.1835-4211.24.2014.1619

Prevention and Treatment of Koala Retrovirus (KoRV) Infection: Lessons from Studies of AIDS Viruses in Nonhuman Primate Models

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Abstract. The presence of multiple retroviruses in koalas (*Phascolarctos cinereus*), including viruses with exogenous infectious forms that may be associated with malignant disease manifestations, poses challenges for both management of captive populations and species preservation in the wild. The development of antiretroviral medications (ARV) for the treatment of human immunodeficiency virus (HIV) infection is one of the triumphs of modern medicine, and many of these drugs have relatively broad antiretroviral activity, suggesting they might be active against koala retroviruses (KoRVs). However, accumulating experience with the use of these medications in non-human primate (NHP) models of HIV infection and acquired immune deficiency syndrome (AIDS) points out several caveats and provides guidance in attempting to use anti-HIV drugs in the treatment of retroviral infection in nonhuman species. This manuscript reviews that experience from the perspective of potential use of ARVs for prevention and treatment of KoRV infection.

Lifson, Jeffrey D. 2014. Prevention and treatment of koala retrovirus (KoRV) infection: lessons from studies of AIDS viruses in nonhuman primate models. In *The Koala and its Retroviruses: Implications for Sustainability and Survival*, ed. Geoffrey W. Pye, Rebecca N. Johnson and Alex D. Greenwood. *Technical Reports of the Australian Museum, Online* 24: 65–69.

The koala (*Phascolarctos cinereus*) represents a fascinating instance of retrovirus/host species interactions, with geographically high prevalence of an endogenizing retrovirus, provisionally designated koala retrovirus-A (KoRV-A), that is also found in exogenous, pathogenic forms, along with a more recently described distinct exogenous related virus, provisionally designated KoRV-B, that utilizes a different cellular receptor and is associated with malignant hematologic manifestations (Ávila-Arcos et al., 2013; Canfield et al., 1988; Hanger et al., 2000; Oliveira et al., 2007; Shojima et al., 2013; Simmons et al., 2012; Stoye, 2006; Tarlinton et al., 2005, 2006, 2008). These viruses represent a management problem for captive populations, and a challenge for species preservation in the wild. The development of antiretroviral drugs for the treatment of HIV infection has dramatically improved both survival and quality of life for HIV infected individuals, and the relatively broad antiretroviral activity of many of these drugs suggest they may also be active against retroviruses affecting non-human species, such as KoRVs (Oliveira et al., 2007). However accumulating experience with the use of anti-HIV drugs in NHP models highlights important considerations and potential limitations to such use that may help inform efforts to use anti-HIV drugs for the treatment of KoRV infection in koalas (Del Prete & Lifson, 2013). Factors to consider include potency against the target virus (compared to HIV), drug delivery, pharmacokinetics, toxicity and sustainability of treatment. Perhaps the most important consideration is the relationship between the mechanism(s) of action and targets of the drugs considered in relation to the underlying pathogenesis of the disease process of concern.

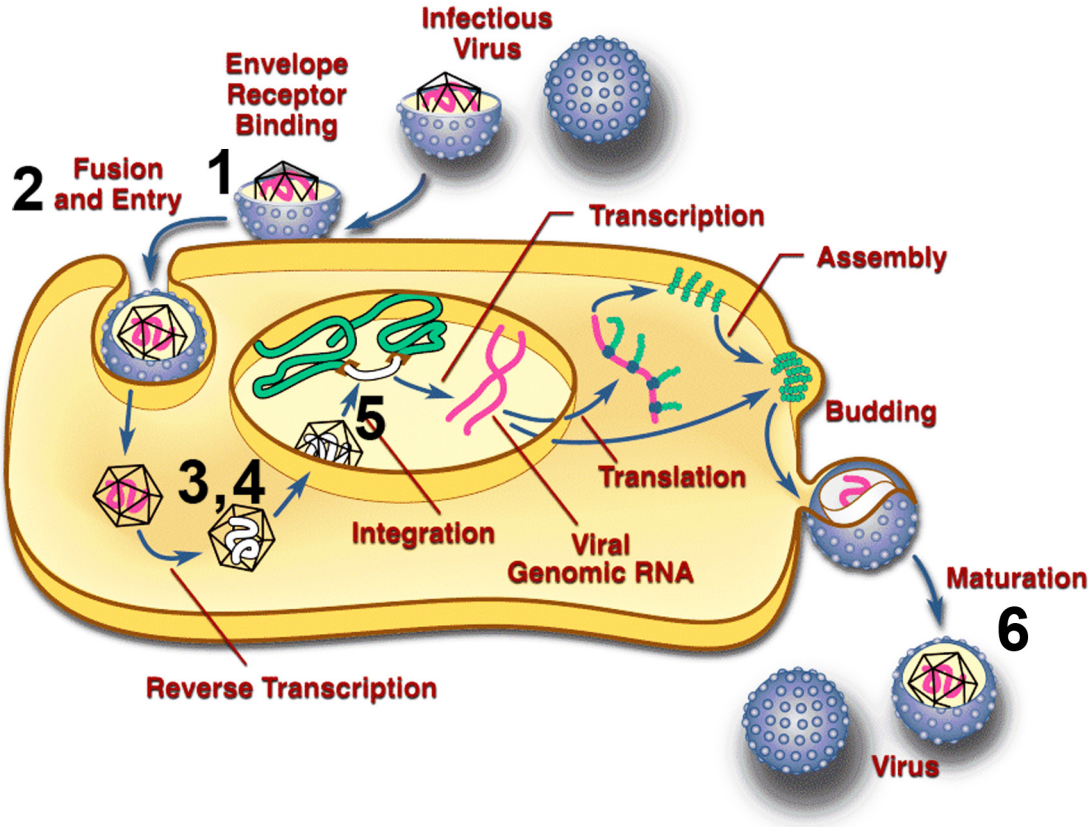

Figure 1. Steps in the retroviral replication cycle present opportunities for therapeutic intervention. Modified from: http://home.ncifcrf.gov/hivdrp/RCAS/images/replication.html

Retroviral replication cycle and drug targets

Retroviruses utilize a host cell dependent, multistep replication cycle for their reproduction that involves extensive interactions with host cell systems (Bieniasz, 2012). Multiple steps in this replication cycle, illustrated in Figure 1, provide potential opportunities for therapeutic intervention, and over the past circa 25 years, a substantial research enterprise has sought to better understand and exploit the therapeutic opportunities presented by these steps.

The retroviral replication cycle is reviewed in detail elsewhere (Bieniasz, 2012). Key steps however are illustrated in Figure 1. (Steps in this replication cycle that are the targets of approved anti-HIV drugs are indicated by numbers in Figure 1): Steps include: binding of mature, cell free virions to receptors and co-receptors on the surface of a susceptible target cell (1), leading to conformational changes that enable facilitated fusion of the membranes of the virion and the host cell (2), entry of the virion contents into the cytoplasm, reverse transcription of the viral RNA genome into DNA (3,4, reflecting two different drug classes targeting the HIV reverse transcriptase), integration of the reverse transcribed viral DNA into host cell chromosomes (5), transcription of viral genes from the resulting integrated provirus, translation of the transcribed viral sequences to produce viral proteins, including viral structural proteins required for virion formation, virion assembly at the membrane of the infected host cell with packaging of viral genomic RNA, budding of virions, with release of immature viral particles, and extracellular maturation of the virions, through cleavage of the viral *gag* protein mediated by the viral protease to yield mature, infectious virions (6). Additional steps in the replication cycle are being targeted in preclinical research in progress.

As of 2012, there were 30 different drug preparations approved by the US FDA for the treatment of HIV infection, including 23 distinct active pharmaceutical ingredients from seven different classes, acting via six different targets, including several fixed dose multidrug combination formulations (reviewed at http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/ucm118915.htm).

Use of anti-HIV drugs in nonhuman primate models of HIV infection and AIDS

Experience with the use of these drugs in the prevention or treatment of AIDS virus infection in NHP models has recently been reviewed (Del Prete & Lifson, 2013). The mainstay of treatment in these models has been the use of the nucleoside analog reverse transcriptase inhibitors (NRTI) tenofovir and emtricitabine, typically given as a daily subcutaneous injection, along with more variable regimen components comprised of oral or subcutaneously administered integrase strand transfer inhibitors (IN-STI) and/or protease inhibitors (PI), with occasional use of co-receptor blockers. While there has been considerable success in the use of anti-HIV drugs for the prevention and treatment of infection in such models, the cumulative experience has also identified some areas which indicate that direct extrapolation from human clinical experience may fail to identify specific challenges inherent in attempting to use these drugs in retrovirally infected animals of other species. There may be significant differences even between macaque species. Some of these challenges are outlined below.

Activity/Potency. Because of species specific viral restriction factors that limit the replication of HIV in NHP cells, most NHP models of HIV infection do not use HIV, but instead use various isolates of the related Simian Immunodeficiency Virus (SIV) or laboratory created chimeric viruses (Bieniasz, 2012; Hatziioannou & Evans, 2012). Many anti-HIV drugs are active against these simian viruses, but this cannot be assumed. For example, although many drugs in the NRTI class work well against simian viruses, their potency against simian viruses may be less than against HIV, and drugs of the non-nucleoside RT inhibitor (NNRTI) class, which act via a different mechanism than NRTIs to inhibit reverse transcriptase (RT), show virtually no activity against the RTs of the simian viruses. Some drugs, such as fusion inhibitors or co-receptor blockers, whose activity is specific to sequences in the HIV viral envelope glycoprotein or the co-receptors used by HIV, would not be expected to have activity against viruses having significantly different envelope glycoprotein sequences and using different receptor systems to gain access to target cells. Even for drugs that are active against simian viruses, the activity of other mechanistic classes of anti-HIV drugs, such as PIs and IN- STIs, is typically less against SIV enzyme targets than against the corresponding HIV enzymes they were developed to inhibit. Thus, despite the potent anti-HIV activity of many different antiretroviral drugs, their activity against other viruses in nonhuman species should not be assumed but must be empirically validated, in suitable in vitro assays, and ultimately in vivo. This is especially true for drugs that require intracellular metabolic activation for pharmacologic activity, such as the intracellular phosphorylation of NRTIs to their phosphorylated pharmacologically active forms. Ideally, in vitro testing should be done using cells of the relevant species, as such metabolic activation may vary between different target cells particularly if derived from different species.

Drug delivery. For sustained administration of ARVs to NHP, the two routes of administration that have been used most extensively are oral delivery and subcutaneous injection. For oral administration to NHP, drugs are generally mixed with food or dietary "treat" items. Challenges in this mode of administration include palatability/acceptance, compatibility of some drugs with different food items based on factors such as pH, the requirement to rotate the food item in which the drugs are presented to avoid boredom and eventual lack of acceptance, along with the relatively resource intensive requirements for staff time for preparation of the drug-in-food mixtures and monitoring to ensure complete consumption each dose (directly observed therapy), particularly for any medications that must be given more than once per day. Differences in oral bioavailability for different drugs, and between animals for the same drug are also important considerations, ideally addressed by monitoring blood levels. Oral absorption of drugs such as PIs and IN-STIs with poor aqueous solubility can be challenging. Many of these factors may be particularly challenging for administration of ARVs to koalas where the restricted dietary options may limit choices for oral administration of drugs, although anecdotal experience suggests that the IN-STI raltegravir can be effectively administered short term when given in eucalyptus flavored Portagen ® (C. Stadler, pers. comm.).

NHP can be behaviorally conditioned to accept subcutaneous injections, and for many drugs that are available in suitable formulations, this is a preferred method of administration. Compared to oral administration, subcutaneous administration is faster and more convenient, requires less staff time, and ensures full bioavailability. Important considerations include the volume to be injected, which in turn depends on the solubility of the drug(s) being injected, compatibility of the drugs included in multidrug combinations, and ensuring that the formulation does not induce any local injection site reactions, especially with sustained dosing. Daily subcutaneous administration of ARVs has been maintained for years in some NHP settings (Van Rompay et al., 2006, 2008, 2012). Work on development of long acting, sustained release formulations of anti-HIV drugs, including nanoformulated preparations, offers promise for more convenient dosing regimens in the future (Baert et al., 2009). Daily subcutaneous injections in koalas can be challenging, but anecdotal experience suggests that at least short term, daily administration of the NRTI tenofovir is feasible (C. Stadler, pers. comm.).

Pharmacokinetics. To maintain viral suppression, and avoid the selection of drug resistant mutant virus, it is important to maintain therapeutic levels of ARVs, particularly at the minimum concentration trough between doses ([C_{min}]). Drug levels are affected by absorption, and metabolism, and species differences in these parameters can affect pharmacokinetics, influencing drug levels over time. Indeed, even between different species of macaques, oral bioavailability of the same drug may vary. Drug metabolism may vary between species, and this may be particularly important for orally administered drugs such as many PIs and IN-STIs that in NHP require twice daily dosing to maintain therapeutic levels, typically defined as plasma levels in excess of the plasma adjusted IC_{95} for virus inhibition in vitro in a relevant assay, that is the drug concentration required for 95% inhibition of viral replication in the presence of plasma which contains proteins that can bind many drugs.

Administration of ARVs to koalas is complicated for orally administered agents by the restricted dietary options for this species and potentially by differences in absorption from a gastrointestinal tract quite different than that of primates (Stupans, 2001). In addition, for both orally and subcutaneously administered drugs potential differences in metabolism between koalas and primates may impact drug levels, emphasizing the desirability of pharmacokinetic analysis of drug levels to empirically determine dosages and administration schedules.

Safety/Tolerance/Toxicity/Drug-Drug interactions. While ARVs are administered to millions of people who in general tolerate them well, toxicities have been clearly identified and well described, particularly for the more commonly used agents. In NHP studies, the ARV related toxicity that has been best established is renal toxicity associated with acute or chronic overdosage with tenofovir, characterized by increased blood urea nitrogen and creatinine, and hypophosphatemia, with histologic findings of acute tubular necrosis and bone pathology, findings similar to tenofovir related toxicities described in humans (Calza, 2012; Sanders-Beer et al., 2011; Van Rompay et al., 2006, 2008, 2012). When multiple drugs are administered concurrently, the potential for drug:drug interactions must be addressed, with the realization that due to differences in drug metabolism drug:drug interactions may vary between species.

Sustainability. An important consideration for long term therapy is the sustainability of treatment, with multiple factors contributing. These include long term tolerance of the administered drugs and mode of dosing, but also maintaining the resources long term to source and administer the drugs.

Relation of drug target/activity to pathogenetic mechanisms. While all of the above considerations are important in contemplating the potential use of ARVs in KoRV infected koalas, arguably the most important consideration is the relation of the target of the drug and its mechanism of action to the pathogenetic mechanisms underlying the disease manifestation of concern. With ARV treatment of HIV infected humans or SIV infected NHP, the available licensed drugs all act to block new rounds of infection with no effect on already infected cells. This approach is efficacious in HIV and SIV infection because of the nature of the pathogenesis mediated by these viruses. In untreated HIV or SIV infection, the majority of viral replication is derived from de novo infection of CD4+ T cells that have a short life span once infected ($T_{<1/2>}$ approximately 1 day) (Wei et al., 1995). Thus, blockade of new rounds of infection substantially reduces overall viral replication levels and the immune activation that is associated with pathogenesis, including loss of CD4+ T cells and disease progression. However, even maximally suppressive ARV treatment of HIV infected patients does not affect virus production from already infected cells or impact latently infected cells. Thus, even prolonged ARV treatment producing maximal suppression of viral replication does not cure HIV or SIV infection as virus persists in cell populations not susceptible to ARV drug suppression, providing a source for recrudescent virus and progressive infection if ARV treatment is stopped (Richman et al., 2009). This has engendered a search for novel strategies beyond ARVs to effect HIV eradication or functional cure (Richman et al., 2009).

The details of the pathogenetic mechanisms underlying hematolymphoid malignancies in KoRV infected koalas remain to be fully elucidated. However, based on precedent from malignancies associated with other gammaretoviruses, it is likely that the underlying pathogenesis, once malignant disease is established, does not rely on de novo infection of new uninfected cells, and that high levels of plasma viremia reflect virus production from already infected cells (Bolin & Levy, 2011). In this situation, ARVs that block new rounds of de novo infection are unlikely to impact viral replication or disease processes. Indeed, anecdotal experience suggests that short term treatment of a KoRV-A/KoRV-B coinfected koala with a combination of a NRTI and an IN-STI did not meaningfully impact plasma viremia levels (C. Stadler, pers. comm.). And ARVs will not be expected to have any impact on endogenized virus, although they may help limit spread to new target cells of infectious forms potentially produced from endogenized sequences.

Thus, the potential applicability of ARVs to KoRV infection may be limited to certain situations. For example, treatment of infected dams and joeys may prevent transmission or pathogenesis of exogenous infectious forms of KoRV, an application well established for HIV and SIV (Mofenson, 2003). It is also possible that early ARV treatment may limit the replication and spread of exogenous infectious forms, potentially preventing viral integrations that may result in malignant transformation of target cells through insertional mutagenesis. However, such treatment might need to be sustained for life, and this prospect is likely not feasible with current drugs and delivery methods.

Alternatives to ARVs for prevention of KoRV infection

If ARVs may have a limited role in combating KoRV infection, what other interventions may be useful? While it has proven extraordinarily challenging to develop effective vaccines for the prevention or control of infection with lentiviruses like HIV or SIV, this is not the case for gammaretroviruses, where efficacious vaccines for feline leukemia virus (FeLV) have been developed (Hoover et al., 1996). This suggests that vaccines for other gammaretroviruses like KoRV-A and KoRV- B should be feasible. Such vaccines should help prevent transmission of exogenous infectious forms of KoRV-B, and potentially provide protection from pathogenesis from infectious forms expressed from endogenized KoRV-A. While a variety of approaches have been employed to develop candidate vaccines against FeLV, an approach that takes advantage of conserved features in the nucleocapsid (NC) proteins of all true retroviruses may be useful in developing a KoRV vaccine. The zinc finger motif in the NC proteins of all true retroviruses is present in KoRV (Shojima et al., 2013; Thomas & Gorelick, 2007). Site directed mutagenesis studies in HIV, SIV, and other retroviruses have shown that maintenance of an intact, authentic retroviral zinc finger motif in NC is required for completion of the viral replication cycle and NC has been implicated as a critical participant in multiple steps of retroviral replication (Thomas & Gorelick, 2007). Chemical treatments that preferentially covalently modify the free sufhydryl groups of the retroviral zinc finger motif in viral NC proteins result in elimination of infectivity, while preserving structurally and functionally intact envelope glycoproteins on the surface of treated virions (Arthur et al., 1998; Rossio et al., 1998). Such chemically inactivated retroviral virions have been shown to be useful vaccine immunogens in other retrovirus systems, and may merit evaluation as a candidate KoRV vaccine (Lifson et al., 2004).

Acknowledgments. This work has been funded in whole or in part with Federal funds from the National Cancer Institute, National Institutes of Health, under Contract No. HHSN261200800001E. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government

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