Inefficient vaginal transmission of tenofovir resistant HIV-1

Morgan Chateau, Michael D. Swanson, and J.V. Garcia*

Division of Infectious Diseases, Department of Internal Medicine, Center for AIDS Research
University of North Carolina, Chapel Hill, North Carolina, USA

*To whom correspondence should be addressed at:
Division of Infectious Diseases
UNC Center for AIDS Research
University of North Carolina School of Medicine
Genetic Medicine Building 2044
Chapel Hill, NC 27599-7042
Tel: 919-843-9600
Fax: 919-966-6870
Email: victor_garcia@med.unc.edu

Running Title: Inefficient vaginal transmission of tenofovir resistant HIV

Abstract Word Count: 88
Text Word Count: 1594
ABSTRACT

Transmission of drug resistant HIV has been postulated to be a threat to current first line antiretroviral therapy (ART) regimes and the efficacy of several antiretroviral based pre-exposure prophylaxis (PrEP) strategies being tested. Here we evaluated the effect of the common tenofovir (TFV) resistance mutation, K65R on vaginal HIV transmission. Our results demonstrate that despite no overt loss of overall replication competence \textit{in vivo}, this mutation results in significantly reduced mucosal transmission. When transmitted the mutant virus eventually reverted to wild type in 2 of 3 animals examined.

TEXT

In absence of a cure or vaccine, and despite valuable efforts toward better HIV education including safe sex practices, the HIV epidemic continues to grow at a faster pace than the current availability of antiretroviral therapy (ART). For every two people who begin ART, five are newly infected (31). Of the people infected, only 47% have access to ART in low and middle income countries (32). There is a great need to prevent transmission of HIV. To address this need, extensive efforts are being made to develop and implement effective pre-exposure prophylaxis (PrEP) approaches. So far, the greatest progress has been made using antiretroviral drug based treatment as prevention and PrEP (1, 9). When the patient has a positive diagnosis and access to a full ART regime under a doctor’s guidance, then early treatment is exceedingly effective for preventing transmission of HIV to uninfected partners (9). Unfortunately, a significant number of HIV+ individuals do not know their infection status, especially during acute infection when transmission potential is highest, increasing the need for alternatives such as PrEP. Most current PrEP clinical trials are investigating the use of antiretroviral drugs either singularly or as a two drug combination for systemic or topical use (1, 2, 30, 33, 34). This raises
an important concern with the dual use of antiretroviral drugs for both treatment and prevention: the consequences of the development and transmission of drug resistant HIV. HIV-1 develops resistance to virtually all drugs currently available for treatment (4, 20).

For this reason, current ART therapies consist of a cocktail of multiple drugs with different classes of action to prevent or at least postpone the development of drug resistant HIV within the patient’s lifespan. Drug-resistant viruses can be transmitted (6, 17, 27, 38) . During new infections certain mutations like M184V are rarely detected by routine genotyping but significantly higher proportions can be detected using more specific methodology (6, 27, 38). The inherent ability of replicating HIV to revert to a drug sensitive genotype in the absence of drug pressure makes it difficult to study in patients especially if: (1) the time/duration/route of infection are unknown, (2) there is no way to prove ART naïve status, and (3) the HIV sequence in the infecting partner is unknown. Despite these difficulties, genotypic analysis of ART naïve patients has provided evidence that drug resistant HIV-1 is being transmitted and can result in treatment failure (3, 5, 15, 18, 22, 24, 25). Given that animal studies are the best option to overcome the inherent limitations of human studies (11), we utilized humanized mice to investigate in vivo transmission of a drug resistant HIV-1.

Tenofovir (TFV) is the drug most commonly used in clinical trials evaluating systemic and topical PrEP. Tenofovir disoproxil fumarate, the oral formulation of TFV, is also part of every DHHS recommended first line therapy (14). For this reason, we chose to study transmission of a tenofovir resistant HIV. The mutation of the lysine at amino acid position 65 in HIV reverse transcriptase to an arginine (K65R) confers resistance to tenofovir as well as other NRTIs. For this reason K65R is on both the WHO and IAS surveillance lists for HIV genotyping (4, 20). There is clinical evidence that HIV containing the K65R mutation can be transmitted.
after mucosal exposure albeit at lower frequency than other mutations like M184V (3, 6, 24, 27).

To evaluate the role of this single amino acid mutation on mucosal HIV transmission, we introduced the K65R mutation (AAA to AGA) into a proviral clone of HIV-1JR-CSF (21). In addition, to differentiate the mutant virus from the parental clone after reversion, a second, silent mutation (TAT to TAC, Tyrosine) was included to act as a molecular marker.

To confirm a decrease in the susceptibility of the mutant virus to TFV, we determined the in vitro IC$_{50}$ for wild type HIVJR-CSF and the isogenic mutant, HIVJR-CSF K65R. The K65R mutation conferred a 4.7 fold increase in the in vitro IC$_{50}$ for TFV, which is comparable to the 2 to 4 fold range reduction in susceptibility reported (16, 37) (Figure 1). Previous in vitro studies have shown that the K65R mutation reduces the function of viral reverse transcriptase (36, 37). It is unknown to what extent this defect affects viral replication in vivo. To test the in vivo replication capacity of HIVJR-CSF K65R, humanized mice (12, 23) were inoculated via IP injection 3x10$^4$ TCIU and viral load in plasma was monitored over time (13). Longitudinal analysis of plasma viral load showed no difference in the in vivo replication of the K65R and wild type strains (Figure 2) in this group of five animals, suggesting that there are not large differences in the in vivo fitness of the mutant virus. Sequence analysis of plasma virus RNA from HIV-1JR-CSF K65R infected mice confirmed the presence of the K65R mutation 2 weeks post infection. However, subsequent time points showed a population of wild type virus. Sequence analysis indicated that reversion of the K65R mutation was always to the original sequence. It should be noted that the molecular marker, present only in the mutant virus, served to exclude the possibility of contamination with wild type virus.

Having demonstrated the replication capacity of the K65R mutant virus in vivo, we next evaluated its capacity to transmit mucosally. For this purpose, we utilized BLT humanized mice.
(13). The female reproductive tract of BLT mice is reconstituted with all the cells relevant for HIV transmission including human T cells, monocyte/macrophages and dendritic cells (13, 28). BLT mice were vaginally exposed once to equal infectious doses of wild type HIV-1JR-CSF or the isogenic K65R mutant virus (3.5X10^5 TCIU). Three independent exposures (n=4) were performed on three different dates. The results of these vaginal exposures showed a dramatic decrease in the transmission efficiency of the K65R mutant virus (Figure 3). Specifically, whereas all the mice exposed to the wild type virus were infected (4/4) only 25% of the mice exposed to the mutant virus were infected (3/12). This difference in vaginal HIV transmission was highly statistically significant by log rank analysis (p=0.011, Mantel Cox). These results demonstrate that the K65R mutant is vaginally transmitted at a greatly reduced rate compared to the wild type virus. Interestingly, these results seem at odds with those recently published by Cong et al (11) using SIV. However, these could due to the facts that a different mutation was used and that additional fitness compensatory mutations were introduced into the provirus used by Cong et al (11).

To determine if the transmitted virus contained the K65R mutation, plasma viral RNA was sequenced at different times after exposure. Four weeks post exposure we noted the presence of only mutant virus in one mouse (M1), the presence of only wild type (reverted) virus in a second mouse (M2), and the presence of both mutant virus and wild type (reverted) virus populations in a third mouse (M3). Longitudinal analysis of the virus found in the plasma of one of the infected mice (M3) showed the presence of both mutant and wild type viruses at weeks 4 and 6 post infection and the presence of wild type virus at all subsequent time points (Table 1). Cervicovaginal lavage (CVL) from this mouse also showed the presence of both wild type and mutant virus 4 weeks post infection. Subsequently only the wild type virus was found in the
CVL (Table 1). Analysis of the virus present in the different tissues from two of the infected mice generally reflected what was observed in the periphery. However, in one mouse the mutant virus was found in the plasma but all tissues analyzed contained both the wild type and mutant viruses. Interestingly, analysis of the virus present in tissues 14 weeks post infection showed the wild type virus in all tissues except thymus where both drug resistant and wild type virus were found (Table 1). These results are consistent with the hypothesis of Weinberg et al suggesting that transmitted viruses that contain reversible mutations become archived in lymphocyte reservoirs (38).

In summary, the topical or systemic use of antiretroviral drugs for the purpose of preventing HIV acquisition has the potential to curtail the spread of AIDS and some PrEP strategies have shown great promise (2, 9, 19, 33, 35). The fact that tenofovir is a successful first line drug for the treatment of HIV infection has made this compound the drug of choice for most prevention trials (10). However, this dual use approach is not without risk as there is significant potential to expand the pool of drug resistance in communities utilizing PrEP (8, 19). Here we test K65R mutated HIV-1 in humanized mice and found that, as in humans, the HIV carrying the K65R mutation is (1) replication competent (Figure 2); (2) is present in cervicovaginal secretions (Table 1); and (3) reverts to wild type in the absence of drug selection although the mutant virus remains detectable (Table 1). Finally, we tested the ability of K65R mutant HIV to transmit vaginally and found that it could transmit, albeit at a significantly lower efficiency than wild type (Figure 3). At this point the molecular basis for this lower transmission are not known. However, analysis of the K65R mutant has shown that it has a decreased replication capacity in comparison to wild type in several in vitro model systems (7, 29). Overall, our results demonstrate that if this tenofovir resistant virus is present in the transmitting partner, there is the
potential for the mutant virus to be transmitted to the uninfected partner with lower efficiency compared to wild type HIV-1.

ACKNOWLEDGEMENTS

This work was supported in part by: U19 AI082637 Combination HIV Antiretroviral Rectal Microbicide (CHARM) Program (MLC), the University of North Carolina Center for AIDS Research (P30AI50410), grant AI73146 (JVG) and National Institutes of Health training grant T32CA9156-37 (MDS). We would like to thank Ian McGowan, Peter Anton and Charlene Dezzutti for reading the manuscript and providing valuable suggestions. We thank Dr. I. Chen for providing the JR-CSF plasmid via the AIDS Research and Reagent Program. We would like to thank former and current lab members and veterinary technicians at UNC Division of Laboratory Animal Medicine for their assistance with various technical aspects of this work.
REFERENCES

   Maarschalk, N. Arulappan, M. Mlotshwa, L. Morris, D. Taylor, and o. b. o. t. C. T.
   Group. 2010. Effectiveness and Safety of Tenofovir Gel, an Antiretroviral Microbicide, for

   Tappero, E. A. Bukusi, C. R. Cohen, E. Katabira, A. Ronald, E. Tumwesigye, E. Were,
   Mucunguzi, E. Nakku-Joloba, R. Twesigye, K. Nguere, C. Apaka, H. Tamooh, F.
   Gabona, A. Mujugira, D. Panteleeff, K. K. Thomas, L. Kidoguchi, M. Krows, J. Revall,
   S. Morrison, H. Haugen, M. Emmanuel-Ogier, L. Ondrejcek, R. W. Coombs, L.
   Frenkel, C. Hendrix, N. N. Bumpus, D. Bangsberg, J. E. Haberer, W. S. Stevens, J. R.
   Lingappa, and C. Celum. 2012. Antiretroviral Prophylaxis for HIV Prevention in

   Fehr. 2011. Minority K65R variants and early failure of antiretroviral therapy in HIV-1-

   Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009


Scribner, and H. Hu. 2008. The prevalence of transmitted antiretroviral drug resistance in


Wensing, and D. D. Richman. 2011. 2011 update of the drug resistance mutations in HIV-
1. Top Antivir Med 19:156-64.

1987. Dual infection of the central nervous system by AIDS viruses with distinct cellular

Shikuma, V. A. Johnson, S. A. Fiscus, R. T. D’Aquila, B. R. Schackman, E. P. Acosta,
inhibitors predicts virologic failure of an efavirenz-based regimen in treatment-naive HIV-1-

23. Lepus, C. M., T. F. Gibson, S. A. Gerber, I. Kawikova, M. Szczepanik, J. Hossain, V.
Ablamunits, N. Kirkiles-Smith, K. C. Herold, R. O. Donis, A. L. Bothwell, J. S. Pober,
and M. J. Harding. 2009. Comparison of human fetal liver, umbilical cord blood, and adult
blood hematopoietic stem cell engraftment in NOD-scid/gammac-/-, Balb/c-Rag1-/-


35. Veronese, F., P. Anton, C. V. Fletcher, V. DeGruttola, I. McGowan, S. Becker, S.


FIG 1. Introduction of K65R mutation into HIVJR-CSF results in a 4.7 fold increase of in vitro IC₅₀ using a luciferase based assay in TZM-bl indicator cells. Serial dilutions of tenofovir were applied to indicator cells in triplicate and allowed to incubate for 30 mins before an equal number of tissue culture infectious units (TCIU) of either wild type or mutant virus was applied to all wells. Two days later, the media is removed, ONE-GLO reagent (Promega) was added and the amount of luciferase activity was measured. Each curve was normalized to wells infected with that specific virus (wild type or K65R virus) in the absence of drug. RLU= relative light units.

FIG 2. In vivo replication of HIVJR-CSF and HIVJR-CSF K65R after IP injection into humanized mice shows no overt difference in replication capacity. Humanized NOD/SCID/gamma⁻/⁻ mice (12, 23) were infected with equal amounts of either HIV-1JR-CSF or HIV-1JR-CSF K65R (3x10⁴ TCIU) by IP injection. The course of infection was monitored by determining plasma viral loads.

FIG 3. The K65R mutation reduces vaginal transmission efficiency of HIV-1 by 75%. Humanized BLT mice were prepared and validated as previously described (13, 26, 28). Mice were exposed vaginally to a single dose of HIV-1JR-CSF or HIV-1JR-CSF K65R (3.5x10⁵ TCIU). Infection was monitored as a function of viral load in plasma. Kaplan-Meyer plot represents the percentage of HIV negative mice as a function of the number of weeks post-exposure until the first peripheral blood HIV-1 detection. In 3/12 (25%) mice viral load was readily detectable 2 weeks post exposure. In 9/12 (75%) mice exposed to the K65R mutant no viral load was detected.
at any time point analyzed and no viral DNA was found in tissues at harvest confirming lack of transmission.
Figure 2

Viral RNA (Copies per mL)

Time (Weeks Post Exposure)
Figure 3

Percent HIV Negative
After Vaginal Exposure

Time (Weeks Post Exposure)

JR-CSF K65R (n=12)
JR-CSF WT (n=4)
p=0.0112
TABLE 1. Sequence analysis demonstrates reversion of the K65R mutation over time in peripheral blood, cervicovaginal lavage and tissues of infected BLT mice*.

<table>
<thead>
<tr>
<th>Vaginally infected by HIVJR-CSF K65R</th>
<th>Week post exposure</th>
<th>Analysis of Viral Sequence from Bulk PCR Products at Position 65</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>M1</td>
<td>4</td>
<td>R only</td>
</tr>
<tr>
<td>M2</td>
<td>4</td>
<td>K only</td>
</tr>
<tr>
<td>M3</td>
<td>4</td>
<td>K and R</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>K and R</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>K only</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>K only</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>K only</td>
</tr>
</tbody>
</table>

*Bone marrow/liver/thymus mice were exposed once intravaginally once to mutant virus. Infection was monitored in plasma by determining viral load. Two mice were harvested four weeks post infection (M1 and M2) and one was harvested 14 weeks post exposure (M3). Peripheral blood and vaginal lavage samples from this mouse were collected longitudinally. Only FRT, female reproductive tract. K, lysine. R, arginine. PCR primer sets used to amplify RT: outer/first reaction: 5'-GCTCTATTAGATACAGGAGC-3', 5'-
CCTAATGCATATTGTGAGTCTG-3', inner/second reaction: 5'-
GTAGGACCTACACCTGTAAC-3', 5'-CCTGCAAAGCTAGGTGAATTGC-3'. Amplification products were sequenced in bulk.