Perinatal exposure of patas monkeys to antiretroviral nucleoside reverse transcriptase inhibitors induces genotoxicity persistent for up to 3 years of age

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ABSTRACT

Background: *Erythrocebus patas* (patas) monkeys were used to model use of antiretroviral (ARV) drugs in HIV-1 infected pregnant women.

Methods: Pregnant patas dams were given human-equivalent daily ARV dosing for 50% of gestation. Mesenchymal cells, cultured from bone marrow of patas offspring obtained at birth, 1 and 3 yr of age, were examined for genotoxicity including: centrosomal amplification (CA); micronuclei (MN); and MN containing whole chromosomes (MN+C).

Results: Compared to controls, significant increases ($p<0.05$) in CA, MN and MN+C were found in most groups of offspring examined at birth, 1 and 3 yr.

Conclusions: Transplacental NRTI exposures induced fetal genotoxicity persistent for 3 yr.
INTRODUCTION

Antiretroviral (ARV) drug combinations provide a highly-successful approach for preventing the transmission of HIV-1/AIDS [1] in approximately 99% of the 10,000 HIV-1-infected women who become pregnant yearly in the United States. The nucleoside reverse transcriptase inhibitors (NRTIs) are major components of the ARV combinations in clinical use. In addition, they become incorporated into nuclear and mitochondrial DNA of the host, resulting in arrest of viral DNA replication [2], chromosome damage [3-5] and genomic instability [6].

Transplacental studies in mice showed incorporation of AZT into fetal organ DNA, mutagenesis, and tumor induction in the offspring [4, 7, 8]. Patas monkeys, born to pregnant dams receiving AZT, had AZT-DNA incorporation in nuclear and mitochondrial DNA of multiple organs [5] that was similar in magnitude to that observed in cord blood and umbilical cord DNA of human fetuses [2]. Furthermore, NRTI-induced mutagenicity was reported in human infants exposed in utero and during the perinatal period [2, 9], suggesting that these infants may be at risk for cancer induction [10].

To observe the fetal consequences of NRTI use in human pregnancy we employed pregnant patas dams exposed to clinically-relevant ARV drug combinations in human-equivalent protocols. Some of the offspring were taken at birth and others were given the same drugs for the first 6 weeks of life, and taken at 1 and 3 years of age. The patas placentation and NRTI pharmacokinetics are similar to those found in humans [11], and because the patas cannot be infected with simian immunodeficiency virus, drug effects can be examined in the absence of virus.
MATERIALS AND METHODS

Patas maintenance, drug sources and exposure protocols

Maintenance of the patas has been previously detailed [12, 13]. Monkeys were housed and treated under conditions approved by the American Association for the Assessment and Accreditation of Laboratory Animal Care International, using protocols reviewed by the Institutional Animal Care and Use Committee of Bioqual, Inc., or the NCI Animal Care and Use Committee. Euthanasia was performed in accordance with the 2007 American Veterinary Medical Association Guidelines on Euthanasia. Monkeys were housed in groups of one male with three females, and pregnancy was assessed by ultrasound. Pregnant dams (n=2-3/group) were given NRTIs during the final 50% (10 wk) of gestation. Exposed and unexposed patas offspring were either taken near-term by cesarean section [13], or born naturally, and grown to 1 or 3 years of age. To obtain unexposed 3 year old patas controls, cells were aspirated from bone marrow of 3-4 year old patas.

Zidovudine (AZT) was from Sigma Chemical Co., (St. Louis, MO), and lamivudine (3TC), the pediatric liquid clinical formulation, was from Glaxo-Wellcome (Raleigh, NC). Abacavir (ABC) and nevirapine (NVP) clinical formulations came from the NIH Veterinary Pharmacy (Bethesda, MD). Each drug was dissolved in syrup that was placed inside of a banana, or mixed into pudding, and given to the pregnant patas, under supervision, as a treat. AZT and ABC were given for the last 10 weeks of gestation, in two 20 mg doses, for a total of 40 mg/day, 5 days/week. 3TC and NVP were given for the last 4 weeks of gestation in two 12 mg doses, for a total of 24 mg/day, 5 days/week. Unexposed monkeys received bananas containing syrup twice daily for 10 wk. To model human clinical ARV use, where infants
receive NRTI therapy for 6 wk after birth, neonates to be observed for 1 and 3 years were dosed with liquid formulation twice daily per os by syringe for the first 6 weeks of life [12].

**Bone Marrow Culture**

Using sterile technique, bone marrow was collected from the femur cultured in T75 flasks (BD Falcon, Bedford, MA) with RPMI 1640 media and 10 or 20% inactivated fetal bovine serum (American Type Culture Collection, ATCC, Manassas, VA). Harvested cells were incubated undisturbed for 48 hrs, at which time the media was changed and colonies of adherent mesenchymal-derived cells were allowed to expand. At confluence, cells were removed by 0.05% Trypsin (Sigma-Aldrich, St Louis MO), and 5,000 or 10,000 cells/chamber were transferred to 4- or 8-well chamber slides (BD Falcon), respectively. Quadruplicate wells were used per treatment group.

**Pericentrin and -Tubulin Staining for Centrosomal Amplification**

The pericentrin immunostaining has been previously described [14]. Anti-pericentrin (Covance, Emeryville, CA, 1:300 dilution) or anti- -tubulin (Sigma-Aldrich, St. Louis, MO, 1:200 dilution), were incubated for 2 hrs at room temperature. Secondary antibodies, anti-rabbit Alexa 488 (Invitrogen, Carlsbad, CA) for pericentrin, and anti-mouse rhodamine red for -tubulin (Invitrogen), were added for 30 min. DNA was visualized (blue) by staining with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI, Invitrogen). Cells stained with -tubulin were red, and those stained with pericentrin antibody showed punctate green signals throughout the cytoplasm. A total of 1,000 cells with visible centrosomes was scored for each treatment.

**Micronuclei (MN) with and without Whole Chromosomes**

Cell monolayers were stained using DAPI (Invitrogen) to localize nuclei and MN. To visualize entire chromosomes in micronuclei, an anti-kinetochore antibody was used to stain
centromeres. Cells were fixed with methanol, permeabilized for 4 min with 0.1% Triton-x100 in PBS, and incubated overnight at 4°C with human anti-kinetochore calcinosis, Raynaud's phenomenon, esophageal dysfunction, sclerodactyly, telangiectasia (CREST) antibody (Antibodies Incorporated, Davis, CA) at a 1:40 dilution in blocking solution. Slides were washed as above and incubated for 90 min at room temperature with an anti-human Alexa 488 (Invitrogen, 1:500 dilution) secondary antiserum in blocking solution (10% goat serum, 0.01% Sodium azide, 1% Bovine serum albumin in PBS). DAPI was used to visualize nuclei and micronuclei. Scoring of 5000 cells per treatment allowed identification of micronuclei with or without CREST-positive green signals.

**Fluorescent and Confocal Microscopy**

Cells were observed using a Nikon Eclipse E-400 microscope (Nikon, Inc, Melville, NY) fitted with a Plan Apo 100x objective with a 1.40 numerical aperture. Stained cells were photographed on a Zeiss Axiovert 100M microscope equipped with a Zeiss Plan Apochromat 100x/1.4 oil Dichromic objective. Confocal images were captured using a Zeiss LSM 510 scanning laser microscope. The LSM 510 zoom software was used to achieve a final magnification of 2000x. Images shown are 3-dimensional maximal projections, which were generated from a series of images through the Z-plane.

**Statistical Evaluation**

For each animal, 1000-5000 cells were examined per end point, and the averages from each animal were grouped by treatment (n=2 to 3 monkeys/group). Comparisons among treatment groups were performed using the Student’s t Test.
RESULTS

Centrosomal Amplification

Mesenchymal fibroblasts (see representative Figure 1A) were cultured from bone marrow, and centrosomes were visualized in fixed cells using either a pericentrin antiserum with a fluorescent tag (green) (Figure 1B and C) or a -tubulin antiserum with a rhodamine red tag (not shown). Figure 1B shows representative cells from an unexposed patas infant with only 1 centrosome per cell (arrow). In contrast, Figure 1C shows multiple centrosomes (circle) in a cell from a patas infant exposed to AZT/3TC. Quantification of CA (Table 1) showed an 8-fold increase in % of cells with CA for AZT/3TC-exposed patas at birth, compared to unexposed patas at birth ($p = 0.0009$). Similarly, in 1 yr patas exposed to AZT/3TC/ABC there was a 6-fold increase in %CA compared to the 1 yr unexposed controls ($p = 0.0001$, Table 1). Finally, in AZT/3TC/ABC-exposed patas at 3 yr there was a 4-fold increase in %CA, compared to the unexposed controls ($p = 0.0006$, Table 1). In contrast, AZT/3TC/NVP-exposed patas at 3 years of age (Table 1) showed no significant increase in % of cells with CA.

Micronuclei

Micronuclei (MN), portions of genetic material separated from the nuclei, are revealed as small blue extranuclear circles by staining with DAPI (Figure 1D, circle). The % of cells with MN, in AZT/3TC-exposed patas at birth, was significantly-higher than in unexposed controls ($p = 0.04$, Table 1). At 1 yr of age, after in utero exposure to AZT/3TC/ABC, there was a 10-fold increase in % MN, compared to the unexposed controls ($p < 0.0001$, Table 1). Cells from AZT/3TC/ABC-exposed patas at 3 yr did not show a significant increase in MN (Table 1). In contrast, a 4.4-fold increase in % MN was observed in cells from AZT/3TC/NVP-exposed patas at 3 years of age, compared to age-matched controls ($p = 0.0009$, Table 1).
Micronuclei Containing Whole Chromosomes

Positive staining with the CREST antiserum is indicative of the presence of kinetochore or centromeric material. Because the centromere is required for chromosomal replication, positive CREST staining within a MN corresponds to presence of a whole (or functional) chromosome. In Figure 1D arrows point to CREST-positive regions, or centrosomes within a nucleus. Aneuploidy results in the corresponding nucleus, when MN contain a CREST signal (Figure 1E, arrow) contain a whole chromosome.

For cells cultured from unexposed and AZT/3TC-exposed patas taken at birth, the % of cells with MN+C (Table 1) was 3-fold higher in NRTI exposed patas compared with the unexposed patas ($p = 0.009$). Patas taken at 1 year of age after in utero exposure to AZT/3TC/ABC (Table 1) showed a 27-fold increase in %MN+C, compared to age-matched unexposed controls ($p = 0.0005$). In addition, cells from AZT/3TC/ABC-exposed patas at 3 years of age ($p = 0.05$, Table 1) and from AZT/3TC/NVP-exposed patas at 3 years of age ($p = 0.003$, Table 1) both showed significant increases in MN+C, compared to the unexposed controls.

Additional comparisons and statistical differences

The statistical relationships in this study, summarized in Table 1, show some additional informative comparisons. For example, values for %CA, %MN and %MN+C, in cells taken from unexposed patas, were similar at all time points, suggesting that there is no increase in these biomarkers during the first 3 yr of life. However, patas offspring exposed to ARV drugs had significant increases in almost all of these biomarkers at the times investigated, with the exception of %MN at 3 yr in infants exposed to AZT/3TC/ABC, and %CA at 3 yr in infants
exposed to AZT/3TC/NVP. Significantly-lower values for %CA, %MN and %MN+C were observed in 3-year old offspring treated with AZT/3TC/ABC, compared to their 1 year counterparts (Table 1), showing a reduction in the number of damaged cells over time. Also, at 3 years of age monkeys exposed to AZT/3TC/ABC had fewer MN than those exposed to AZT/3TC/NVP ($p=0.01$), suggesting that AZT/3TC/NVP is the more persistently-genotoxic combination. Finally, the overall values for %MN+C are lower than those for %MN, because the loss of a whole chromosome is a more rare event than the partial loss of genetic material.

**DISCUSSION**

Here we have shown, using an ex vivo approach, that genomic instability and chromosome loss, induced in patas monkey bone marrow by in utero and perinatal exposure to human-equivalent protocols of ARV drugs, is persistent up to 3 years of age and can be revealed by measuring biomarkers of genotoxicity. The data showed significantly-increased levels of %CA, %MN and %MN+C in most ARV drug-exposed groups, compared to unexposed animals, at birth, 1 and 3 years of age. The damage in 1 and 3 year old patas, where the drug combinations were given *in utero* and for the first 6 weeks of life, revealed the persistence of this damage over time. Overall, these experiments reveal that, in addition to the direct mutagenic effects caused by NRTI-DNA incorporation, the NRTIs damage DNA indirectly by producing centrosomal and spindle abnormalities resulting in aneuploidy.

These patas transplacental studies have been designed to model human clinical protocols, and the daily doses given are close, although for 3TC and NVP the duration of exposure was shortened to 4 wk instead of 10 wk. Interestingly, the companion paper to this study, by Andre-Schmutz et al. [15] describes karyotyping of cord blood CD34$^-$ cells from ARV-exposed and unexposed infants, in which significantly more aneuploid cells were found in ARV-exposed
infants born to HIV-1-infected mothers, than in unexposed infants from uninfected pregnancies. Taken together the patas and human studies provide a powerful statement for induction of fetal hematopoietic genotoxicity by in utero and perinatal ARV-drug exposures, especially since a 3 year old patas monkey is developmentally-similar to a 14 year old human. It may be important to follow HIV-1-uninfected children, born to HIV-1-infected mothers, long-term to evaluate the potential for persistent genotoxicity.

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FOOTNOTES

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ABBREVIATIONS

ABC, Abacavir
ARV, antiretroviral
AZT, zidovudine, 3'-azido-3'-deoxythymidine
CA, centrosomal amplification
CREST antiserum, antiserum against calcinosis, Raynaud’s phenomenon, esophageal dysfunction, sclerodactyly and telangiectasia, which stains kinetochore material
DAPI, 4’,6-diamidino-2-phenylindole dihydrochloride
ddi, Didanosine, 2’,3’-didehydro-2’,3’-dideoxythymidine, Videx
bw, body weight
HIV-1, human immunodeficiency virus-1
3TC,lamivudine, (-)β-L-2’, 3’-Dideoxy-3’-thiacytidine
MN, micronuclei, fragments of nuclear material extruded from the nucleus
MN+C, micronuclei containing whole chromosomes
NRTI, nucleoside reverse transcriptase inhibitor
NVP, Nevirapine
PBS, 8.1 mM Na₂HPO₄, 1.9 mM KH₂PO₄, 137.0 mM NaCl, 2.7 mM KCl
PBST, PBS with 0.05% Tween 20
REFERENCES


**FIGURE LEGEND**

**Figure 1** – Photos showing representative examples of: (A) mesenchymal fibroblasts grown from patas bone marrow; (B) unexposed patas fibroblasts with centrosomes (pericentrin staining) shown in green (arrow); (C) fibroblast from AZT/3TC exposed patas showing CA (circle); (D) large nucleus stained with DAPI (blue) showing centromeres stained with CREST antiserum in green (arrow) and micronucleus stained with DAPI (circle); (E) large patas nucleus from NRTI exposed animal with adjacent micronucleus containing whole chromosomes (CREST-positive green dots, arrow).
Table 1 – Comparison of values for %CA, %MN and %MN+C (mean ± SE), among patas treatment groups.

<table>
<thead>
<tr>
<th>Age</th>
<th>Exposure (n)</th>
<th>%CA</th>
<th>% Total MN</th>
<th>%MN + C</th>
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<tbody>
<tr>
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<tr>
<td>Birth</td>
<td>Control (3)</td>
<td>0.57 ± 0.35</td>
<td>1.14 ± 0.16</td>
<td>0.28 ± 0.09</td>
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<td></td>
<td>AZT/3TC (3)</td>
<td>4.50 ± 0.27</td>
<td>2.34 ± 0.36</td>
<td>0.83 ± 0.07</td>
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<tr>
<td></td>
<td></td>
<td><em>P=0.0009</em></td>
<td><em>P=0.04</em></td>
<td><em>P=0.009</em></td>
</tr>
<tr>
<td>1 year</td>
<td>Control (3)</td>
<td>0.74 ± 0.05</td>
<td>0.58 ± 0.07</td>
<td>0.11 ± 0.06</td>
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<tr>
<td></td>
<td>AZT/3TC/ABC (2)</td>
<td>4.40 ± 0.18(^a)</td>
<td>6.23 ± 0.13(^a)</td>
<td>3.02 ± 0.22(^a)</td>
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<tr>
<td></td>
<td></td>
<td><em>P=0.0001</em></td>
<td><em>P&lt;0.0001</em></td>
<td><em>P=0.0005</em></td>
</tr>
<tr>
<td>3 years</td>
<td>Control (3)</td>
<td>0.49 ± 0.11</td>
<td>0.70 ± 0.21</td>
<td>0.18 ± 0.15</td>
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<td>AZT/3TC/ABC (3)</td>
<td>1.90 ± 0.09(^a)</td>
<td>1.47 ± 0.36(^a, b)</td>
<td>0.72 ± 0.19(^a)</td>
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<td><em>P=0.0006</em></td>
<td><em>P=0.13</em></td>
<td><em>P=0.05</em></td>
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<tr>
<td>3 years</td>
<td>Control (3)</td>
<td>0.49 ± 0.11</td>
<td>0.70 ± 0.21</td>
<td>0.18 ± 0.15</td>
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<td>AZT/3TC/NVP (3)(^c)</td>
<td>3.75 ± 1.55(^c)</td>
<td>3.11 ± 0.18(^b)</td>
<td>1.09 ± 0.14</td>
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<tr>
<td></td>
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<td><em>P=0.07</em></td>
<td><em>P=0.0009</em></td>
<td><em>P=0.003</em></td>
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</table>

\(^a\) Differences for AZT/3TC/ABC at 1 year vs. 3 years are significant to *P=0.0008, 0.002, and 0.0004*, for %CA, %MN and %MN+C, respectively, demonstrating reduction in AZT/3TC/ABC genotoxicity between 1 and 3 years of age.

\(^b\) At 3 years of age, values for %MN in cultured bone marrow cells from monkeys exposed to AZT/3TC/ABC were significantly lower than those exposed to ABC/3TC/NVP (*P=0.01*).

\(^c\) For %CA there were 2 monkeys for this group.