

The Effect of Tenofovir on Bone Remodeling

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Dedication

To my family, thank you for your continued love and support.

Abstract

With the introduction of Highly Active Anti-Retroviral Therapy (HAART), HIV-infected patients have had long-term survival rates increase dramatically. A significant sequela from HIV-infection and side effect from some HAART medications is the loss of bone mineral density. The purpose of this study was to investigate the effects of a common HAART medication, tenofovir, on bone cells. It was hypothesized that tenofovir alters the gene expression of osteoblasts and osteoclasts, which creates an imbalance in the bone remodeling system and skeletal change. To test this, mice were treated with tenofovir, then serum was obtained to determine changes in osteocalcin, osteopontin, and carboxyl telopeptides of type I collagen activity. Furthermore, changes in osteoclast numbers were obtained by tartrate resistant acid phosphatase staining of mouse femurs. While other studies have shown that gene expression is altered in tenofovir treated cells, the present study did not find a significant difference in osteocalcin, osteopontin or carboxyl telopeptide of type I collagen activity for tenofovir mice. However, tenofovir decreased the number of osteoclasts in tartrate resistant acid phosphatase stained sections in mice. Given that HIV patients taking tenofovir medication have an increase in osteoporosis and the results of this study indicate tenofovir alone creates osteopetrotic effect, further studies are needed to determine the relationship between HIV infection and tenofovir.

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INTRODUCTION

Nearly 1 million Americans and approximately 33 million people worldwide are infected with HIV [1,2]. With the introduction of Highly Active Anti-Retroviral Therapy (HAART) in the 1990's, HIV-infected patients have had long-term survival rates increase dramatically. Current reports state that in 2005 24% of HIV patients living in the US were 50 years of age and older, and that number is estimated to grow to over 50% by 2015 [3,4]. With improved survival rates, long term care for HIV-infected patients needs to be established with a focus on geriatric issues. A significant sequela from HIV-infection and side effect from some HAART medications is the loss of bone mineral density, which leads to osteoporosis and increased risk of bone fracture [5,6]. HAART drug classes that have been implicated as risk factors for premature bone loss include reverse transcriptase inhibitors, such as tenofovir, and protease inhibitors [5]. Determining the mechanisms of bone loss in HIV-infected patients is critical to forming successful treatment to prevent or reverse bone disease and improve patient quality of life.

REVIEW OF THE LITERATURE

Bone

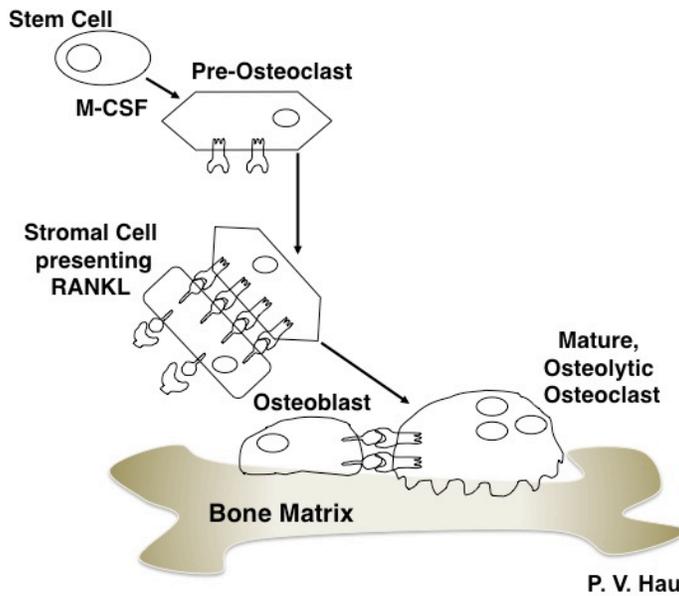
Bone is a dynamic tissue, which is continuously remodeled for normal bone growth and maintenance. It consists of an organic component, which is primarily type I collagen, and an inorganic component, which serves as a reservoir for calcium, phosphate, and magnesium ions [6].

Bone Cells

The two key cell types in bone are osteoblasts and osteoclasts. Osteoblasts build bone by synthesizing collagen and glycoproteins. Osteoclasts resorb bone by secreting lysosomal hydrolases and metalloproteinases, such as collagenase and gelatinase. Intricate cell signaling between osteoblasts and osteoclasts is required for maintaining bone homeostasis [5]. If an imbalance occurs between these cells, bone pathologies, such as osteoporosis, periodontal disease, Paget's disease of bone, and cancer-related bone diseases, may result [6].

Osteoclasts are derived from hematopoietic cells in the bone marrow. Macrophage colony-stimulating factor (M-CSF) is necessary for proliferation and survival of osteoclast precursors and macrophages, and stimulates osteoclast expression of nuclear factor kappa B ligand (RANK). RANK is expressed by osteoclasts and interacts with nuclear factor kappa B ligand (RANKL), which is expressed by osteoblasts (Figure 1). RANKL stimulates osteoclast activity and is regulated in part by a decoy receptor, osteoprotegerin (OPG), also produced by osteoblasts [7]. Local factors such as the cytokines interleukin-1 (IL-1), tumor necrosis factor (TNF), transforming growth factor- β (TGF- β), and interferon- γ , are key regulators of osteoclasts and act through changes in RANKL and OPG. Systemic hormones, such as parathyroid hormone, vitamin D3, calcitonin, and sex steroid hormones, also regulate osteoclast activity either directly or indirectly through osteoblasts [6].

Figure 1: Osteoclast differentiation and activation



Osteoclast resorption of bone releases collagen peptides, pyridinoline crosslink fragments, and calcium from the bone matrix. The collagen peptides and fragments, such as carboxyl telopeptides (CTX), can be measured as

biochemical markers of bone metabolism. Another biomarker for bone metabolism is tartrate-resistant acid phosphatase (TRAP), a lytic enzyme produced by osteoclasts [6].

Osteoblast precursor cells are derived from pluripotent stromal mesenchymal cells. Mature osteoblasts synthesize type I collagen, osteocalcin (OCN), cell attachment proteins (thrombospondin, fibronectin, bone sialoprotein, osteopontin), proteoglycans, and growth-related proteins. Osteoblast differentiation is regulated by transcription factors Runx-2, osterix, and beta-catenin. Several hormones and growth factors alter osteoblast function and activity. Growth factors TGF- β , insulin-like growth factors (IGFs)-I and -II, and platelet-derived growth factor (PDGF) are autocrine regulators of osteoblast function [6].

Highly Active Antiretroviral Therapy (HAART)

Highly Active Antiretroviral Therapy has been very successful in clinically

managing HIV infection. HAART generally includes three agents, two of which are nucleoside or nucleotide reverse transcriptase inhibitors and the other either an HIV protease inhibitor or a nonnucleoside reverse transcriptase inhibitor [8].

Tenofovir

Tenofovir is the most common nucleotide reverse transcriptase inhibitor (NtRTI) used in HAART due to its high efficacy and low side effect profile [9]. The prodrug form is tenofovir disoproxil fumarate (TDF) (Figure 2). NtRTIs and nucleoside reverse transcriptase inhibitors (NRTI) inhibit reverse transcriptase, a viral DNA polymerase enzyme that retroviruses need to reproduce. Normally, NRTIs are converted into nucleotide analogs by the body. Taking NtRTIs directly allows conversion steps to be skipped, which allows for more rapid activation. Other positives of the NtRTI tenofovir

include high potency, low cellular and mitochondrial toxicity, and single daily dosing [10].

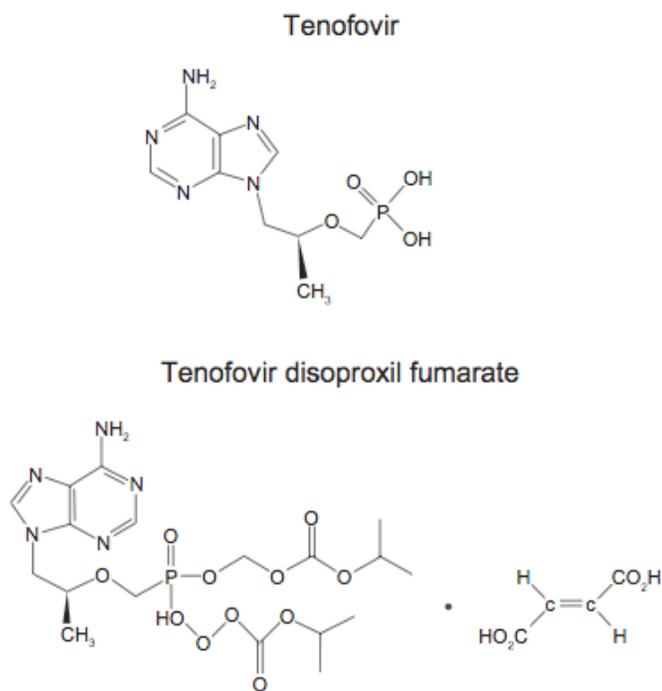


Figure 2: Structures of tenofovir and tenofovir disoproxil fumarate (TDF)

While tenofovir has many benefits as an HIV medication, negative side effects exist. Several studies have indicated a possible link between tenofovir and decreased bone mineral density [5, 11-13]. Clinical studies involving HIV-infected children treated with tenofovir revealed bone abnormalities, such as unfused epiphyses and decreased trabecular bone [14,15]. Tenofovir was associated with a greater decline in bone mineral density compared to a NRTI, abacavir, in a recent clinical trial [16]. Macaque research has shown decreased bone mineral density following administration of tenofovir [14,15,17]. There is also evidence that tenofovir induced bone abnormalities may be involved in oral bone loss [18,19].

Gene expression of primary osteoblasts and osteoclasts following tenofovir administration has been studied *in vitro*. Osteoclasts demonstrated down regulation for Gnas, Got2, and Snord32a genes [20]. Osteoblasts demonstrated altered gene expression for over 70 genes involved in cell signaling, cell cycle, and amino acid metabolism [10]. These *in vitro* studies indicate that tenofovir mediated bone loss could be due, in part, to changes in gene expression that result in osteoclast and osteoblast dysfunction [10,20].

Potential mechanisms for tenofovir effecting bone include: 1. Increased uptake by osteoblasts or osteoclasts, or both, which may alter gene expression and result in a disruption between the balance of osteoclasts and osteoblasts, 2. Tenofovir is a phosphonate and may act like a bisphosphonate, which targets bone and inhibits osteoclast function by inducing apoptosis, 3. Tenofovir-induced renal dysfunction due to renal proximal tubule dysfunction and phosphate wasting [21].

HIV Infection

HIV infects vital cells of the immune system, including macrophages and helper T-cells, creating a progressive failure of the immune system. HIV infection itself has also been implicated as a risk factor for altered bone mineral density [22-25]. Constant stimulation of the immune system by HIV can result in release of inflammatory mediators, such as cytokines [26]. Inflammatory cytokines, such as TNF-alpha, IL-1, and IL-6, can promote osteoclast formation [5].

General risk factors for low bone mineral density in HIV-infected individuals include: low body mass, physical inactivity, malabsorption, and hypogonadism [5,6]. Moreover, HIV-infected individuals have high rates of vitamin D deficiency, and a greater tendency to smoke or abuse drugs and alcohol, which are all general risk factors for osteoporosis [26]. In a recent clinical study, HIV-infected men were shown to be more at risk for osteoporosis when compared to pre-menopausal HIV-infected females of a similar age [27]. The study reports this may be due differences in sex hormone concentrations between males and females, particularly estrogens.

While tenofovir has been implicated as a risk factor for premature bone loss in HIV patients, the independent effects of tenofovir and HIV on bone cells have not been well documented [5]. It is difficult to separate out the exact cause of osteopenia in HIV-infected patients due to the many risk factors typically present, including: HIV-infection and suppressed immune system, numerous drugs given simultaneously, and lifestyle factors. The aim of this study was to determine the independent effects of tenofovir on osteoclasts and osteoblasts using a mouse model with a normal immune system and administering only one HIV medication.

SPECIFIC AIMS

1. Measure osteoclast activity using the biochemical marker carboxy telopeptides collagen type I. Measure osteoblast activity using the biochemical markers osteopontin and osteocalcin.
2. Determine the number of osteoclasts present by tartrate-resistant acid phosphatase staining of mouse femurs.
3. Limit variables that could also be influencing HIV-associated osteoporosis by using a mouse model without an HIV-infection and administering only one medication.

HYPOTHESIS

Tenofovir alters the gene expression of cells involved in bone formation, which effects the activity of osteoblasts and osteoclasts, creating an imbalance in the remodeling system and skeletal change.

MATERIALS AND METHODS

Mice

A total of 11 male C57/Bl6 mice were used in the study. Starting at 21 days of age, the six mice in the experimental group were given injections once a day of 30 mg/kg of tenofovir. Five control mice were injected with phosphate buffered saline (PBS) once a day starting at 21 days of age. All male mice were used to limit the effect of estrogen levels on bone density. The dosage of 30 mg/kg of tenofovir was chosen because it is the equivalent dose given to humans.

Mice were monitored for any adverse reactions, primarily through watching their activity after injection. They were weighed once a week and that weight was used to calculate the dosing of tenofovir or saline. At 3 months of age, mice were euthanized and serum, femurs, and skulls were harvested. The use and care of the mice in this study was approved by the University of Minnesota Institutional Animal Care and Use Committee.

Mouse Osteopontin Immunoassay

Osteopontin (OPN) is a protein secreted by osteoblasts and is deposited into mineralized bone. Increased serum osteopontin levels are directly related to an increase in osteoblast activity. The Quantikine mouse osteopontin immunoassay (R&D Systems, Inc., Minneapolis, Minnesota, USA) was used to measure the amount of OPN in tenofovir and control mouse serum. The kit is commercially available and uses the sandwich enzyme-linked immunosorbant assay (ELISA) technique. The protocol given by the manufacturer was strictly followed.

In brief, a polyclonal antibody specific for mouse OPN was pre-coated onto a microplate. Standard, control, and sample serum, 50 μ L each, were pipetted into the well and any mouse OPN present was bound by the immobilized antibody. The wells were incubated for two hours at room temperature. Any unbound substances were then washed away. Next, 100 μ L of an enzyme linked polyclonal antibody specific for mouse OPN was added to each well and allowed to incubate for 2 hours at room temperature. Wells were then washed to remove any unbound antibody-enzyme reagent. A substrate solution of 100 μ L was added to each well and allowed to incubate, protected from the light, for 30 minutes at room temperature. The enzyme reaction yields a blue product that turns

yellow when 100 μ L of Stop Solution is added to each well. The intensity of the color measured is proportional to the amount of mouse OPN bound in the initial step. Optical density was determined using a microplate reader (Beckman Coulter AB340) set to a wavelength of 450 nm.

A standard curve was constructed using our standards, with known OPN concentrations. A best fit line was then obtained from the standard data (Figure 3), and the equation of the line ($y=mx+b$), where m = slope = 0.0002614, b = y-intercept = 0.006756, y = optical density, was solved for x to determine OPN mouse concentration (pg/mL) for tenofovir and control mice.

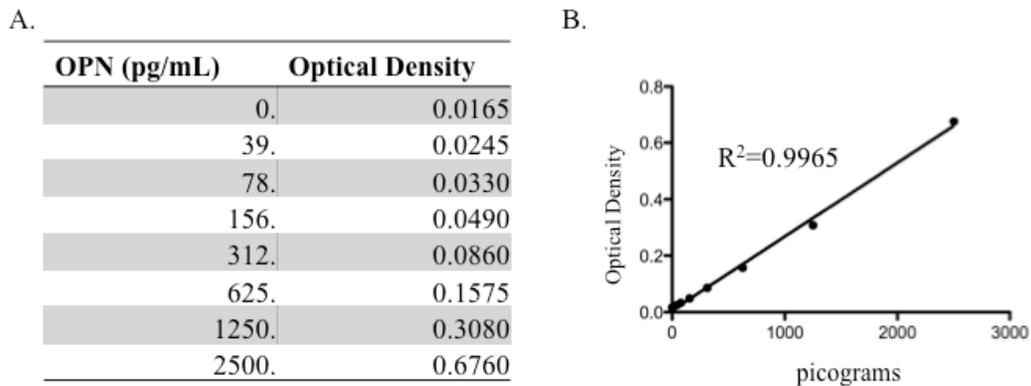


Figure 3: Osteopontin standard curve

Mouse Osteocalcin Immunoassay

Osteocalcin is a non-collagenous protein secreted by osteoblasts during bone formation. Increased serum osteocalcin levels are directly related to an increase in osteoblast activity. The BTI Mouse Osteocalcin EIA kit (Biomedical Technologies Inc., Stoughton, Massachusetts, USA) was used to measure the amount of osteocalcin in

tenofovir and control mouse serum. The kit utilizes the sandwich ELISA technique. The protocol given by the manufacturer was strictly followed.

In summary, two mouse osteocalcin antibodies were used, each directed toward an end of the osteocalcin molecule (C or N terminal). One mouse osteocalcin antibody is bound to the wells, which binds the mouse osteocalcin standard or sample, the other biotinylated antibody completes the sandwich. Standard, control, and sample serum, 25 μ L each, were pipetted into individual wells, followed by 100 μ L of osteocalcin antiserum into each well. The wells were incubated at 2-8 degrees Celsius, 18-24 hours. Any unbound substances were then washed away. Next, 100 μ L of Streptavidin-Horseradish Peroxidase was added to each well and allowed to incubate for 30 minutes at room temperature. A substrate solution of 100 μ L was added to each well and incubated, protected from the light, for 15 minutes. A stop solution of 100 μ L was added to all wells and optical density was read at 450 nm.

As was described for OPN, a standard curve was constructed (Figure 4) which we used to determine concentration of OCN in the serum from control or tenofovir mice.

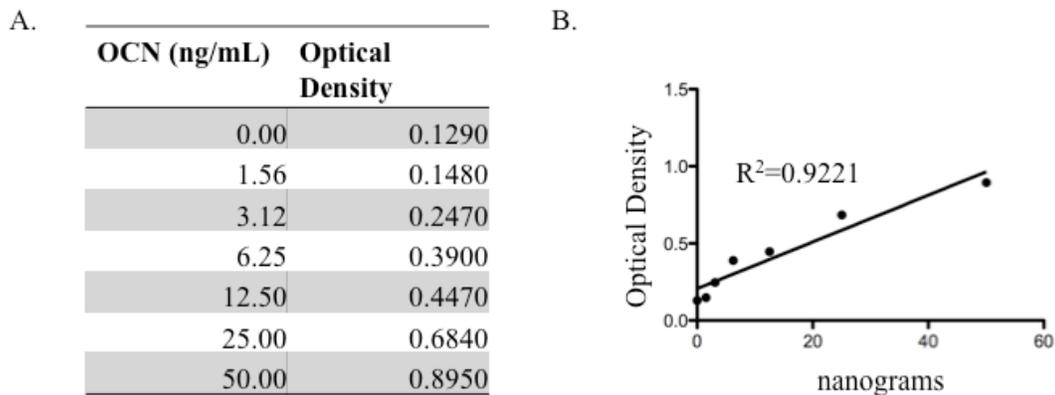


Figure 4: Osteocalcin standard curve

CTX Immunoassay

The RatLaps EIA enzyme immunoassay was used to measure the amount of type I collagen fragments (carboxyl telopeptides or CTX) in mouse serum in tenofovir and control mice, which are released during osteoclast activity. Increased serum CTX levels are directly related to an increase in osteoclast activity. The protocol given by the manufacturer was strictly followed. The RatLaps EIA is based upon the competitive binding of the polyclonal antibody to soluble RatLaps antigens EKSQDGGR or to immobilized RatLaps antigens. The polyclonal antibody is raised against a synthetic peptide having a sequence (EKSQDGGR) specific for a part of the C-terminal telopeptide alpha-1 chain of rat type 1 collagen.

During the pre-incubation step, biotinylated EKSQDGGR is immobilized by binding to the streptavidin-coated wells. The wells are emptied and washed. Standards, control and tenofovir samples of 20 uL each were pipetted into appropriate wells in duplicate, followed by a 100 uL primary antibody solution (polyclonal rabbit), then allowed to incubate 15-21 hours at 2-8 degrees Celsius. Following the primary-incubation step the wells are emptied and washed. In the secondary-incubation step a 100 uL solution of Goat anti-Rabbit antibody conjugated with peroxidase (secondary antibody) is added to each well, which binds to the polyclonal rabbit antibody. After the third washing, 100 uL of a chromogenic substrate is added to each well and incubated for 15 minutes in darkness. The color reaction is stopped with 100 uL sulfuric acid in each well. Finally, the absorbance at 450 nm was measured. The absorbance level is inversely related to the concentration of RatLaps antigens in the sample.

As was described for OPN, a standard curve was constructed (Figure 5) which was used to determine concentration of CTX in the serum from control or tenofovir treated mice.

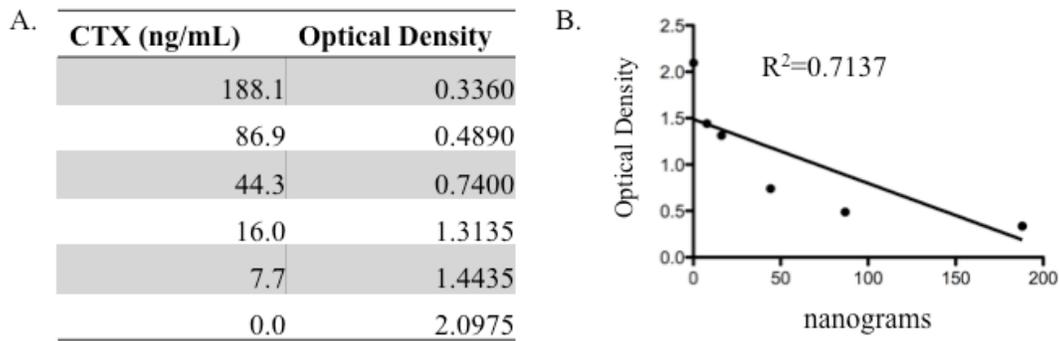


Figure 5: CTX standard curve

Tartrate-resistant acid phosphatase staining

Histological sections from decalcified mouse femurs were stained for TRAP activity using the Leukocyte Acid Phosphatase (TRAP) kit (Sigma-Aldrich, St. Louis, MO, USA). Femurs were from mice treated with tenofovir or PBS for 3 months. Five sections from each mouse were obtained.

In brief, sections were prepared as follows: Femurs were fixed in formalin. Formalin was then replaced with 70% alcohol and femurs were stored at 4 degrees Celsius. Femurs were then sent to the University of Minnesota Comparative Pathology Shared Resource center where they were embedded in paraffin and sectioned. Next, sections were deparaffinized with xylene and hydrated with decreased concentrations of ethanol. Once rehydrated, the sections were TRAP stained strictly following the kit

manufacturer's instructions. Numbers of osteoclasts present in each bone section were counted manually.

STATISTICAL ANALYSIS

Differences in mouse weight, TRAP staining, and osteopontin, osteocalcin, and CTX concentrations, were tested for statistical significance using unpaired t-tests. Sample size was determined by using a power calculation based on previous mouse studies conducted in the Mansky lab. Statistical analyses were performed using GraphPad Prism 5 software with p-values below 0.05 considered statistically significant. The mean and standard error of the mean were calculated for each variable.

RESULTS

Mouse Weight

Average change in mouse weight for tenofovir was 8.8 ± 0.4881 grams, compared to 7.4 ± 0.9214 grams for control. Mouse weight did not differ significantly ($p = 0.2091$) between control and tenofovir treated mice (Figure 6), which suggests that tenofovir injections were not causing low bone mass due to low overall body mass or vice versa.

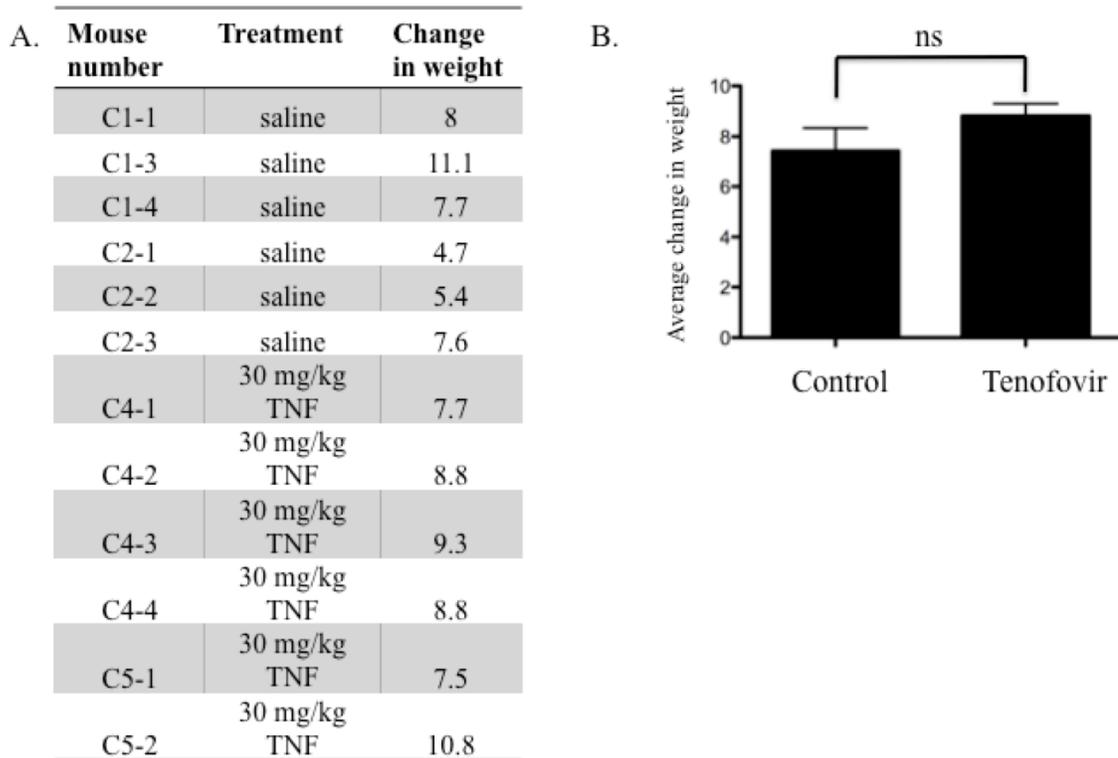


Figure 6. Analysis of Mouse Weight (grams)

Mouse Osteopontin Immunoassay

It was determined there was no significant difference in osteopontin levels for tenofovir versus control mice ($p = 0.0936$) (Figure 7). The average osteopontin concentration for tenofovir mice was 2409 ± 133.3 pg/mL. The average concentration for the control mice was 2849 ± 202.6 pg/mL.

A.

OPN (pg/mL)	
Control	Tenofovir
2118.533	2812.922
2803.358	2338.519
3337.062	2330.867
3044.386	2585.285
2943.001	2535.549
	1850.724

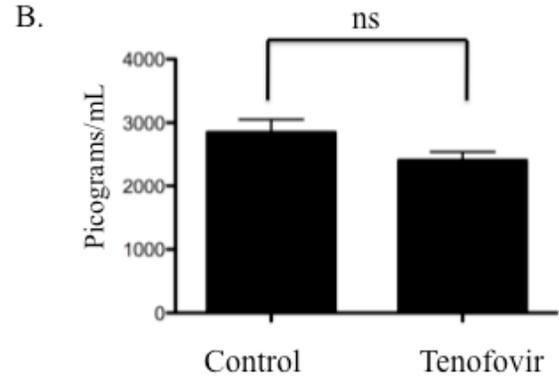


Figure 7: Osteopontin in tenofovir and control treated mice

Osteocalcin Immunoassay

No significant differences were found when the osteocalcin concentration of the tenofovir mice was compared to control mice ($p = 0.7941$) (Figure 8). The average osteocalcin concentration for tenofovir mice was 9.7 ± 2.6 ng/mL. The average concentration for the control mice was 8.5 ± 3.4 ng/mL.

A.

OCN (ng/mL)	
Control	Tenofovir
6.013	2.121
21.450	13.864
4.628	19.999
8.916	9.246
1.594	4.958
	7.795

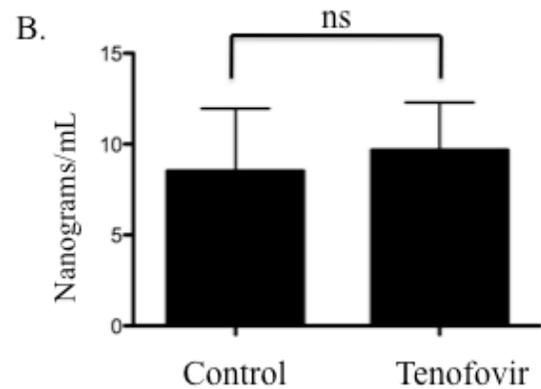


Figure 8: Osteocalcin concentration in control and tenofovir treated mice

CTX Immunoassay

There was no significant difference found between the tenofovir and control mice ($p = 0.7174$) (Figure 9). The average CTX concentration for tenofovir mice was 18.6 ± 3.7 ng/mL. The average concentration for the control mice was 20.6 ± 4.0 ng/mL.

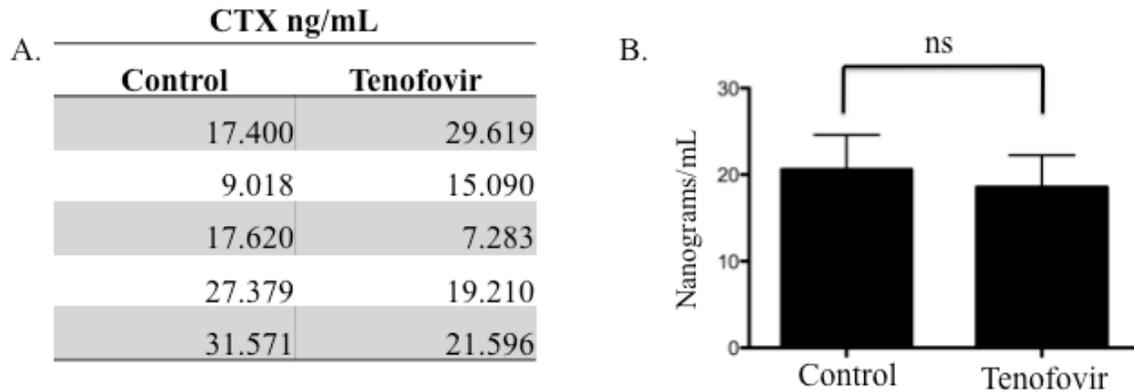


Figure 9: CTX concentration in control and tenofovir treated mice

TRAP Staining of femurs

In tenofovir mice there were significantly less osteoclasts found compared to control mice ($p = 0.0003$) (Figure 10). The average number of osteoclasts for control was 32.6 ± 4.3 while the average number for tenofovir was 9.3 ± 1.2 .

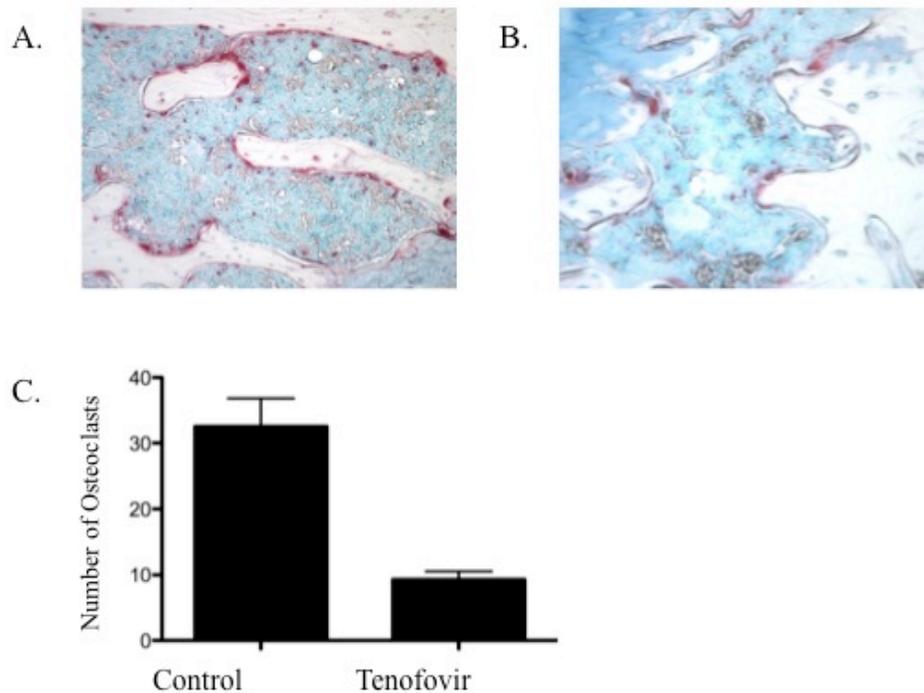


Figure 10: Representative image of TRAP stained mouse femurs. (A) TRAP stained section of femur from control treated mouse. (B) TRAP stained section of femur from 3-month tenofovir treated mouse. (C) In tenofovir mice there were significantly less osteoclasts found compared to control mice ($p = 0.0003$).

DISCUSSION

The present study aimed at determining the independent effects of tenofovir on osteoblasts and osteoclasts using a mouse model. In general, the results show there was no significant difference in osteoblast or osteoclast activity between the control and tenofovir mice. There were however less osteoclasts present in TRAP stained femur sections of tenofovir mice compared to control.

From the results of the present study, overall it appears that giving the mice tenofovir alone causes osteopetrosis due to a decrease in osteoclast number. The study

demonstrates that tenofovir may be acting like a bisphosphonate, which increases bone density by inducing apoptosis of osteoclasts [6]. Pharmacists studying tenofovir have confirmed its chemical structure appears to be similar to a phosphonate (figure 11) (personal communication with Dr. Steve Patterson).

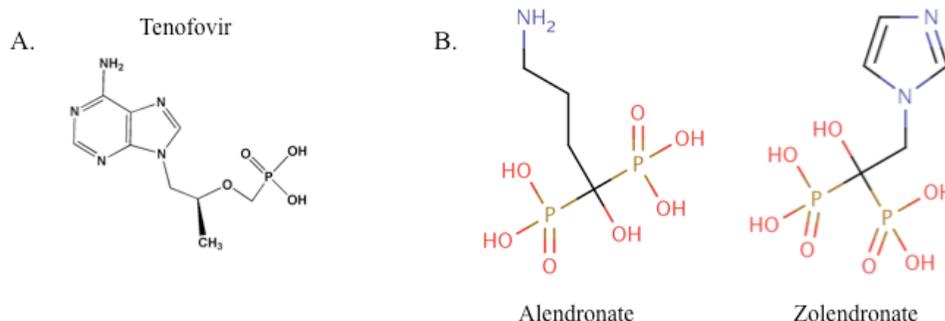


Figure 11: Structure of tenofovir and two commonly prescribed bisphosphonates

However, the CTX data does not coincide with a decrease in osteoclast number, as seen in the TRAP staining data. When reviewing the standard curve and best fit line for the CTX ELISA, the goodness of fit (R^2 value) is low (0.71). The value is low because when the standard values are plotted, they form a curved line, and a straight line must then be estimated from the curved line to determine the equation of a line. To account for possible operator error, the CTX ELISA was repeated, however similar results were found. One other possible reason the CTX data does not correlate with the TRAP data is due to the age of mice used. According to the manufacturer's instructions, the age of mice is critical for measuring CTX activity, and mice that are too old may not have enough CTX activity to distinguish a significant difference between control and

treated mice. Therefore, it may have been beneficial to use younger mice. Another reason the CTX data may not correlate with the TRAP data is because osteoclast activity is related to the number of osteoclasts present, as well as the size of the osteoclasts. The larger the osteoclasts, the more surface area they can resorb, and the more CTX released [6]. Therefore it is possible to have less osteoclasts but the same amount of osteoclast activity, if the osteoclasts are larger than normal.

Recently, several other tenofovir experiments have been performed in the Mansky lab, which do support a decrease in osteoclast activity following tenofovir treatment. Experiments performed include: Microarray analysis, microCT analysis, and real time PCR.

Microarray

Microarray results show altered gene expression for both osteoclasts and osteoblasts. Osteoclasts demonstrated down regulation for *Gnas*, *Got2*, and *Snord32a* genes [20]. Osteoblasts demonstrated altered gene expression for over 70 genes involved in cell signaling, cell cycle, and amino acid metabolism [10].

MicroCT

MicroCT data shows that femurs in tenofovir treated mice are osteopetrotic versus control treated animals.

Real Time Polymerase Chain Reaction

Real Time Polymerase Chain Reaction (PCR) data was obtained by using 1 month and 3 month tenofovir treated and control mouse skulls. Results show a significant

decrease in gene expression in osteoclasts and osteoblasts of tenofovir treated versus control.

While Real Time PCR data demonstrates a significant decrease in gene expression of tenofovir treated mice, the osteopontin, osteocalcin, and CTX ELISAs show no significant difference in osteoblast or osteoclast activity. One reason the real time data may not be consistent with the ELISA data is because of a difference in sample location. Individual bones have different mechanisms and rates of remodeling [6]. Real time PCR measured remodeling activity of one bone type (skull), while the ELISAs measured overall activity of the skeleton (serum). Therefore, when measuring the effect of tenofovir using serum, the average remodeling activity of entire skeleton is measured, which tenofovir may overall have a limited effect. However, bones with high rates of remodeling may be more affected by tenofovir treatment. In the future, real time data should be obtained for several different types of bones.

Given that HIV patients taking tenofovir medication have a decrease in bone density, the present study did not expect to find tenofovir mice to have an increase in bone density. There are several potential explanations for this finding:

First, the mice did not have HIV infection present, and there may be an unknown interaction between tenofovir, HIV-infection, and a suppressed immune system. The independent effects of HIV infection and specific antiretroviral drugs on osteoblasts and osteoclasts have not been well established [5]. The present study was specifically designed to determine the independent effect of tenofovir on bone remodeling cells.

Second, mice were given the human equivalent dose of tenofovir, however, mice have a much higher metabolism than humans. The mice may have needed a higher dose of tenofovir to demonstrate the same effects seen in humans.

Third, the sample size of this study was small. There was a trend towards less CTX activity for tenofovir mice, however an increase sample size may be needed to improve the statistical power of the tests. Sample size was determined using a power calculation based on previous mouse studies conducted in the Mansky lab. However, mice used in the previous studies were genetically altered siblings and consequently more genetically similar than the mice used in this study. This may have contributed to a larger variation among the mice within their respective group, tenofovir or control, than expected in this study.

CONCLUSION AND OUTLOOK

The present results suggest that giving the mice tenofovir alone causes osteopetrosis due to a decrease in osteoclast number. Given that HIV patients taking tenofovir medication have an increase in osteoporosis and the results of this study indicate tenofovir alone has osteopetrotic effect, further studies are needed to determine the relationship between HIV infection and tenofovir. Determining the mechanisms of bone loss in HIV-infected patients is critical to forming successful treatment to prevent or reverse bone disease and improve patient quality of life. Given the HIV patient population is aging, with 24% now 50 years of age or older [4], the long term impact of HIV infection and HAART on bone health needs to be considered.

The next step is to create a humanized HIV mouse model and determine the effects of tenofovir on bone mineral density with HIV present. The humanized HIV mouse model will be used to compare the effects of tenofovir to Abacavir (the second most commonly used NRTI) to an untreated HIV infection. Future study design considerations include: an increase in sample size, increase in tenofovir dosing for mice, evaluation of 1 month and 3 month old mice, evaluation of more than one bone type, and measuring osteoclast size in addition to osteoclast number.

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