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# Downregulation of Gnas, Got2 and Snord32a following tenofovir exposure of primary osteoclasts

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ABSTRACT

Clinical observations have implicated the antiretroviral drug tenofovir with bone density loss during the management of HIV infection. The goal of this study was to investigate the in vitro effects of tenofovir exposure of primary osteoclasts in order to gain insights into the potential mechanisms for the drug-induced bone density loss. We hypothesized that tenofovir may alter the expression of key genes involved in osteoclast function. To test this, primary osteoclasts were exposed to physiologically relevant concentrations of the prodrug tenofovir disoproxil fumarate (TDF), then intensive microarray analysis was done to compare tenofovir-treated versus untreated cells. Specific downregulation of Gnas, Got2 and Snord32a were observed in the TDF-treated cells. The functions of these genes help to explain the basis for tenofovir-associated bone density loss. Our studies represent the first analysis of the effects of tenofovir on osteoclast gene expression and help to explain the basis of tenofovir-associated bone density loss in HIV-infected individuals.



## Introduction

Human immunodeficiency virus (HIV) is a major pandemic, with over 30 million infected people worldwide. Highly active antiretroviral therapy (HAART), which combines several antiretroviral drugs, is effective in managing the viral infection but is associated with viral drug resistance and toxicities. Tenofovir, and its prodrug tenofovir disoproxil fumarate (TDF), is a nucleotide analog reverse transcriptase inhibitor (NtRTI) that competes with deoxynucleoside triphosphates (dNTPs) during HIV reverse transcription, leading to chain termination due to its lacking a 3′-hydroxyl group on the deoxyribose required to form a new 5′–3′ phosphodiester bond that extends the DNA chain. TDF has advantages over the nucleoside analogs used to treat HIV infection. First, the activation of TDF is more rapid compared to that of nucleoside reverse transcriptase inhibitors (NRTIs) since it is phosphorylated, which abbreviates the intracellular activation pathway for a more rapid and complete conversion from the prodrug to the active drug. Second, TDF has minimal cellular and mitochondrial toxicity compared to nucleoside analogs [1–4]. Nonetheless, TDF has been reported to be associated with loss of bone mineral density (BMD), particularly in young children and adolescents [5–8].

Bone homeostasis relies on the balance of bone formation and resorption, which are conducted by osteoblasts and osteoclasts, respectively. Osteoblasts regulate osteoclast differentiation by expressing two factors that are necessary and sufficient for osteoclast formation: M-CSF and RANKL. M-CSF is required for survival and proliferation of early osteoclast precursors. Binding of RANKL and the RANK receptor on osteoclasts stimulates expression of genes necessary for osteoclast differentiation, cellular fusion and bone resorption. Osteoblasts also express osteoprotegerin, a soluble decoy receptor for RANKL, which can inhibit the activation of RANK by RANKL. The ratio of RANKL to osteoprotegerin produced by osteoblasts helps to determine osteoclast forming activity within the bone microenvironment and is involved in the close coordination between bone formation and bone resorption under normal physiological conditions.

For children and adolescents, the rate of bone formation exceeds that of bone resorption, allowing for the size and mass of bone to increase over time. Studies in rhesus monkeys have demonstrated the inhibition of cortical bone mineralization and bone toxicity following the administration of TDF [5,6]. Furthermore, clinical studies have found that TDF therapy in HIV-infected children resulted in bone abnormalities such as unfused epiphyses and decreased trabecular bone [8,9]. TDF-induced bone

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abnormalities such as osteopenia, osteoporosis and spontaneous fractures may lead to the loss of alveolar crestal height and oral bone loss [10,11]. Little is known about the mechanism(s) of TDF-associated bone density loss in HIV-infected individuals.

In this study, we investigated the in vitro effects of tenofovir exposure of primary osteoclasts in order to gain insights into the potential mechanisms for drug-induced bone density loss. We tested the hypothesis that tenofovir may perturb the expression of key genes involved in osteoclast function that would increase bone resorption. To do this, we isolated primary murine osteoclasts, exposed to physiologically relevant concentrations of TDF, and then purified total RNA for intensive microarray analysis. Comparison of TDF-treated versus untreated cells revealed specific downregulation of Gnas, Got2 and Snord32a in the TDF-treated cells. The functions of these genes help support a model to explain the basis for tenofovir-associated bone loss. Our studies represent the first analysis of the effects of tenofovir on osteoclast gene expression and help to explain the basis of tenofovir-associated bone density loss in HIV-infected individuals.

## Materials and methods

*Primary osteoclast cultures, viability, TRAP staining.* Primary osteoclast cultures were prepared from spleen of 5 day old mice as previously described [12–14]. Cell viability was determined by measurement of cellular ATP using the CellTiter-Glo Luminescent Cell Viability Assay using the manufacturer's instructions (Promega, Madison, WI). TRAP staining was done using the TRAcP5b kit (Sigma–Aldrich).

*Microarray analysis.* Primary murine osteoclasts were prepared and treated with 500 nM TDF for 72 h. Total RNA from primary osteoclasts was then extracted using the RNeasy mini plus kit (Qiagen, Valencia, CA). Four independent replicates from TDF-treated primary osteoclasts and untreated cells were analyzed. Two gene chips were used in each replicate experiment. Assistance in RNA quality control, labeling, hybridization, and initial data analysis was provided by Genome Explorations Inc. (Memphis, TN). The integrity of the RNA was examined by capillary electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) with RNA 6000 Neno Lab-in-a-Chip Kit (Agilent Technologies) following manufacturer's instructions. Total RNA was used for cDNA synthesis with the reverse transcription–in vitro transcription (RT–IVT) method [15] using the GeneChip WT cDNA Synthesis and Amplification kit (Affymetrix, Santa Clara, CA) according to manufacturer's instructions.

Fragmented and labeled cDNA was hybridized for 17 h at 45 °C to GeneChip Mouse Gene 1.0 ST Arrays (Affymetrix). The mouse Gene 1.0 ST array is an expression array featuring whole genome-transcript coverage with 750,000 unique oligonucleotide probes representing a total number of 28,853 mouse genes. There were approximately 27 probes covering the full length of each gene. Background correction, normalization, and signal summarization (per probe set) were calculated accordingly [16]. For each transcript, an independent *t*-test (5% confidence) was applied to access significance of expression level based on RMA absolute signal log ratios $\geqslant 1.0$.

*Quantitative real-time PCR.* Primary osteoclasts were treated with TDF, washed in 1× phosphate-buffered saline (PBS) twice prior to RNA extraction. The RNeasy mini plus kit (Qiagen, Valencia, CA) was used to yield total RNA and to remove traces of genomic DNA contamination from cells based on manufacturer's instructions. Immediately following RNA extraction, 500 μg of total RNA from each sample was used to generate first stand cDNA as templates for quantitative PCR (qPCR) with the Transcriptor high fidelity cDNA kit (Roche applied science, Indianapolis, IN). SYBR green qPCR reagents from Invitrogen (Carlsbad, CA) were used to detect real-time gene expression. The GAPDH and 18S rRNA genes were used as internal controls for normalization. Three independent experiments were performed. Sequences of primer sets used are available upon request.

## Results and discussion

The goal of this study was to determine whether in vitro treatment of primary osteoclasts with TDF, the prodrug of tenofovir (Fig. 1A and B), would alter gene expression as determined by microarray analysis, and provide insights into how drug exposure may influence osteoclast function. We chose primary murine osteoclasts for analysis as they provide a readily tractable model system to assess the mechanism(s) involved in tenofovir-mediated bone abnormalities that can be readily translated into mouse models of HIV infection. Such models would allow for the in vivo

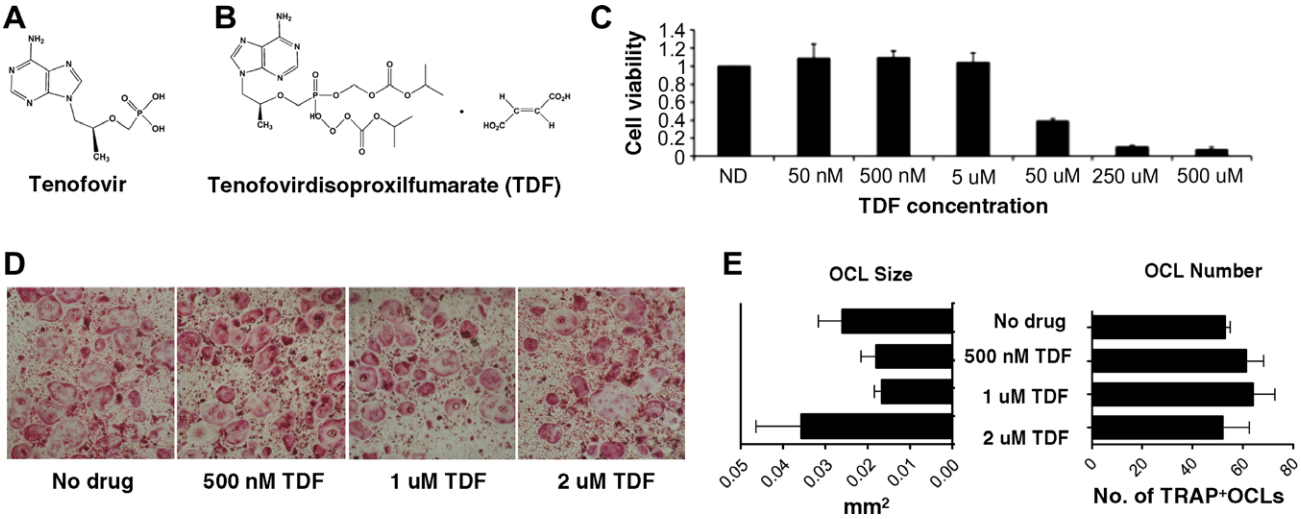


**Fig. 1.** Tenofovir structure and primary osteoclast cell viability following drug exposure. The structure of tenofovir (A) and tenofovir disoproxil fumarate (TDF, the prodrug of tenofovir) (B) is shown. (C) Viability of primary osteoclasts following exposure to TDF. A TDF dilution series was added to primary osteoclast cultures for 72 h, refreshing every 24 h. Cell viability was determined by ATP detection. Tartrate-resistant acid phosphatase (TRAP) staining of untreated (D) and TDF-treated primary osteoclasts and analysis (E) is shown.