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Modulation of Osteoclastogenesis Induced by Nucleoside Reverse Transcriptase Inhibitors

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Abstract

Osteopenia is a common and debilitating side-effect of HAART, yet little is known concerning the effects of HAART on bone metabolism. We reported previously that zidovudine (AZT) stimulates osteoclastogenesis *in vitro* and causes osteopenia in mice. Here, we confirmed that the AZT-induced osteoclastogenesis is dependent on RANKL in that osteoclastogenesis is blocked by osteoprotegerin. Alendronate, which is used for the treatment of osteopenia and osteoporosis, failed to inhibit AZT-induced osteoclastogenesis *in vitro*. Osteoclastogenesis *in vitro* was not affected by tumor necrosis factor- α . Two other NRTI drugs, ddI and 3TC, also induced osteoclastogenesis *in vitro* and induced osteopenia in mice. The osteopenia was associated with an elevation of parameters of osteoclasts, but not with osteoblasts. Combinations of the NRTIs did not result in additive or synergistic effects *in vitro* or *in vivo*. Finally, AZT induced osteoclastogenesis of human osteoclast precursors in a RANKL-dependent manner. This *in vitro* osteoclastogenesis assay using human peripheral blood mononuclear cells could be useful in evaluating bone turnover and the risk of developing osteopenia in AIDS patients on HAART.

INTRODUCTION

The advent of highly active antiretroviral therapy (HAART) has resulted in both a dramatic decrease in the rate of AIDS-related mortality and a significant extension of the life span of HIV-infected individuals. Common complications in HIV-infected individuals are osteopenia and osteoporosis. It is well established that metabolic bone disease is one of the potential side effects of HAART,^{1–3} and the incidence of osteopenia and osteoporosis is higher in HIV-infected individuals receiving antiretroviral therapy than in HIV-infected individuals not receiving antiretroviral therapy.^{2,4–7} As little is known regarding how HAART affects bone metabolism, we decided to analyze the effects of the nucleoside reverse transcriptase inhibitors (NRTIs) as these are a common component of most HAART regimens.

The maintenance of bone density is a dynamic process in which bone loss and bone gain are tightly regulated. Bone remodeling is carried out by osteoclasts, whereas bone is produced by osteoblasts. The overall activity of osteoclasts is defined by the balance in the number of mature osteoclasts that differentiate from osteoclast precursors in response to various signals, and the extent of induction of apoptosis of the osteoclasts triggered by factors such as osteoprotegerin (OPG). The generation of osteoclasts (osteoclastogenesis) involves proliferation, differentiation, cell fusion, and maturation that results in multinucleate cells that express tartrate-resistant acid phosphatase (TRAP) and are capable of resorbing bone.

We reported previously that the NRTI zidovudine (AZT) decreases bone mineral density (BMD) in mice,⁸ thus confirming that AZT can affect bone metabolism independently of the effects of HIV infection. We also showed that AZT enhanced osteoclastogenesis of RAW264.7 cells in a dose-dependent manner, and that this effect required the receptor-activator of NF- κ B ligand (RANKL).⁸ AZT markedly increased the ability of RANKL to stimulate the promoter activity of TRAP and the binding and activity of the nuclear transcription protein, NF- κ B, which has been shown previously to be a key transcription factor in the induction of osteoclastogenesis.^{9–11} It is well established that RANKL is critical to the pathogenesis of many osteoclastogenic disorders. It is expressed by osteoblasts, stromal cells, B-lymphoid lineage cells, and activated T cells^{12–14} under the regulation of hormones such as parathyroid hormone (PTH), $1\alpha,25(\text{OH})_2\text{D}_3$, and prostaglandin E_2 (PGE_2), inflammatory cytokines such as interferon- γ (IFN- γ), various interleukins, and growth factors such as tumor necrosis factor- α (TNF- α). TNF- α is itself an osteoclastogenic cytokine that has been implicated in the development of inflammatory bone erosions^{15,16} and has been shown to enhance the ability of RANKL to induce marrow macrophages to commit to the osteoclast phenotype.¹⁷

OPG is an antiosteoclastogenic factor that binds RANKL and blocks its interaction with RANK. It has been used to treat various metabolic bone and joint disorders caused by an increased number of activated osteoclasts and for diminishing bone pain associated with cancer metastasis.^{18,19} Other antiosteoclastogenic agents include the bisphosphonates, such as alendronate, which are used commonly to treat osteoporosis and cancer-associated bone osteolysis²⁰ and have been used to treat or prevent osteopenia in AIDS patients on HAART.^{21–23} The key mechanisms responsible for the efficacy of bisphosphonates in treating osteopenia and osteoporosis are considered to be inhibition of osteoclast activity and enhanced apoptosis.^{24–26}

In the present study, we confirmed and extended our previous study to the analysis of the effects of other NRTIs, alone and in combination, *in vitro* and *in vivo*. The results indicated that NRTIs act to enhance RANKL-dependent osteoclastogenesis through a mechanism that can be blocked by OPG, but not alendronate (ALN). We further characterized the variables, including cell growth, and TNF- α , on AZT-induced osteoclastogenesis. Although proliferation and differentiation of osteoclasts are enhanced by treatment with NR-TIs, these agents appeared to have little or no effect on apoptosis in the presence of RANKL. Furthermore, no change in osteoblast activity was found in an uninfected mouse model. Neither additive nor synergistic effects of combinations of NRTIs were observed *in vitro* or *in vivo*. We also confirmed that NRTIs stimulate osteoclastogenesis of human peripheral osteoclast precursors in a RANKL-dependent manner. The application of this assay to the analysis of osteoclastogenesis using fresh human PBMCs paves the way for the potential application of this assay as a noninvasive procedure for diagnosis of osteopenia and osteoporosis and prediction of the potential susceptibility of individuals to NRTI-associated metabolic bone toxicity. This is particularly important as not all HIV-infected individuals receiving HAART develop osteopenia or osteoporosis.²⁷

MATERIALS AND METHODS

Reagents

AZT, other NRTIs, ALN, and TRAP assay kits were purchased from Sigma (St. Louis, MO). Stock solutions (1 mM) of NRTIs were prepared as per the manufacturer's instruction, aliquoted, and stored at -20°C . Aliquots were thawed once immediately before use and diluted in Dulbecco's modified Eagle's medium (DMEM) to the appropriate concentration. GST-RANKL (aa 158–316) was produced in our laboratory using the protocol of Lam *et al.*¹⁷ Recombinant murine colony-stimulating factor (M-CSF) was purchased from R & D Systems

Inc. (Minneapolis, MN). Stocks of ALN (100 μ M) were prepared with 0.15 mol/liter NaCl, titrated to pH 7.4 with NaOH, and stored at -20°C .

Cell culture

The mouse macrophage preosteoclast cell line RAW264.7 was purchased from the ATCC (American Type Culture Collection, Manassas, VA) and maintained at 37°C , 5% CO_2 in DMEM supplemented with 100 units/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 10% heat-inactivated fetal calf serum (Hyclone, Logan, UT). For standard osteoclast differentiation experiments, DMEM was replaced with minimum essential medium, α modification (α -MEM), supplemented with 10% fetal calf serum and 50 ng/ml RANKL and cells were cultured at 37°C , 6% CO_2 in a humidified incubator. Medium was changed every 2–3 days.

Preparation of fresh human peripheral blood mononuclear cells (PBMCs)

Fresh human PBMCs were isolated from whole blood. Briefly, 8–10 ml whole blood was diluted by addition of a 2-fold greater volume of Hanks' balanced saline solution (HBSS), layered onto 15 ml Ficoll-Hypaque, and centrifuged (1500g) for 25 min. The PBMCs on the top of Ficoll-Hypaque were then collected,²⁸ washed twice with warm HBSS-EDTA containing 1% fetal bovine serum (FBS, 10 ml), and cultured in DMEM supplemented with 10% FBS, penicillin (100 IU/ml), and streptomycin (100 $\mu\text{g/ml}$) for 1–2 h. The viability of the cells was determined using a 0.4% trypan blue exclusion assay.

Osteoclast differentiation assay (TRAP staining)

Osteoclast formation was measured by TRAP staining of cells plated in 24-well plates as described previously using a leukocyte acid phosphatase assay kit as per the manufacturer's instructions (Sigma).⁸ Osteoclast differentiation was quantitatively determined by measuring TRAP intensity.²⁹ The formation of TRAP intensity in each well was measured as described previously.²⁹ Briefly, the plates were scanned by a transparent light scanner, and the red color image was extracted for PhotoShop (Adobe Systems Inc., San Jose, CA) analysis. The relative intensity of the red color image was then measured using Image (National Institutes of Health) and is referred to as TRAP staining intensity in relative gray.

Osteoclast differentiation was also assessed by reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was isolated from cultured RAW264.7 cells treated with RANKL alone or RANKL plus AZT at the indicated day using Trizol reagent (Invitrogen, CA). RT-PCR was performed as per the manufacturer's instructions (Promega). The primer pairs were as follows:

TRAP,	5'-GGAGCTTAAGTGCCTCTTGCA-3' (forward) and 5'-CACCTCGCTCCTCTGACACT-3' (reverse) ³⁰ ;
Calcitonin receptor (CTR),	5'-TACTGGCTGCACTGTGC-3' (forward) and 5'-ACCCATAATAGCCAGAGAA-3' (reverse) ³¹ ;
GAPDH,	5'-CCCTCACAATTTCATCCA-3' (forward) and 5'-TCCCTAGGCCCTCCTGTTA-3' (reverse).

The reaction products were electrophoresed through a 1.8% agarose gel containing ethidium bromide. The mRNA levels were normalized to GAPDH mRNA levels.

Detection of apoptosis

RAW264.7 cells were cultured with or without AZT (25 μM) in the presence and absence of RANKL (50 ng/ml) for 2 days. Cells were harvested and washed with cold phosphate-buffered saline (PBS) twice. Cells were stained with Annexin V and PI per the manufacturer's

instructions (BD Bioscience, CA). Cells were fixed in 1% paraformaldehyde/PBS and apoptotic and necrotic cells were detected by flow cytometry.

Animals

Male C57BL/6 mice, 4–5 months of age, were divided into three groups of six animals that received vehicle (water) only, AZT only, or AZT plus dideoxyinosine (ddI). The drugs were administered orally using an animal oral feeding needle (Fisher). The doses of NRTIs were calculated based on the doses used in treating HIV-infected individuals^{32–35} and were 0.25 mg AZT, or 0.25 mg AZT plus 0.1 mg ddI, in 0.25 ml water once per day for 4 weeks.

Histology and histomorphometry

Vertebrae were isolated from mice and fixed in 10% buffered formalin, followed by decalcification in EDTA solution for 2 weeks at room temperature with gentle stirring. Vertebrae were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) prior to histopathological examination. Bone histomorphometry was analyzed according to the guidelines of Parfitt *et al.*³⁶ using Bioquant Image Analysis software (R&M Biometrics, Nashville, TN). Histomorphometric measurements, including the ratio of trabecular volume to tissue volume (BV/TV), osteoclast number (OcN/BS), and osteoclast surface area (OcS/BS), were made and are expressed in standard terms. Osteoclast numbers are expressed as the TRAP-positive area per mm² total bone area.³⁷

Osteoclastogenesis system using human PBMC in vitro

PBMCs were plated in a 24-well plate at 5×10^5 /well with 0.5 ml differentiation medium in the presence and absence of AZT and/or other NRTIs for 2–3 weeks. The differentiation medium consisted of α -MEM containing 10% FBS, penicillin (100 IU/ml) and streptomycin (100 μ g/ml), human M-CSF (25 ng/ml), and RANKL (100 ng/ml) and was changed every 3–4 days. Complete α -MEM containing human M-CSF alone without RANKL was used as a control. Each assay was performed in triplicate. At the termination of the experiment, the cultured PBMC were fixed with 10% formaldehyde in PBS for 3 min and subject to TRAP staining (Sigma, St Louis, MO) as per the manufacturer's instructions. The TRAP-positive multinucleated cells containing three or more nuclei were counted.

Statistical analysis

The standard Student's *t*-test was used for statistical analysis of *in vitro* experiments and a one-way ANOVA for comparing values from two groups of mice in the *in vivo* experiments. $p > 0.05$ was considered the limit of statistical significance.

RESULTS

Effect of OPG and ALN on AZT-enhanced osteoclastogenesis

Our previous studies indicate that AZT enhances osteoclastogenesis in a RANKL-dependent manner.⁸ To confirm this, RANKL-stimulated RAW264.7 cells were cultured with or without OPG (100 ng/ml) in the presence and absence of AZT and osteoclastogenesis measured using the TRAP staining assay. The increased osteoclastogenesis induced by RANKL or RANKL plus AZT was almost completely blocked by OPG (Fig. 1). This is of particular interest as OPG has been used to treat osteoporosis and other metabolic bone disorders.

ALN, another osteoclast inhibitor, is used to inhibit bone resorption, increase bone mass and prevent fractures.^{38,39} The precise mechanism(s) of ALN remains unclear, although it is thought to act by stimulating apoptosis of osteoclasts, inhibiting osteoclast activity or recruitment.^{40,41} To determine the inhibitory effect of ALN on AZT-enhanced

osteoclastogenesis, RAW264.7 cells were cultured with the indicated concentrations of ALN ($0 \sim 10^{-6}$ M) in the absence and presence of RANKL plus AZT for 4 days. We found that increased osteoclastogenesis induced by RANKL plus AZT ($p < 0.05$ compared to RANKL alone, Fig. 3) was not inhibited by ALN at low concentrations ($10^{-9} \sim 10^{-8}$ M). However, a high dose of ALN ($\geq 10^{-7}$ M) exhibited a slight inhibitory effect that failed to reach statistical significance between RANKL and RANKL plus AZT ($p > 0.05$, Fig. 2). Treatment with the ALN alone, either at a low dose or high dose, did not induce osteoclast differentiation (data not shown).

Effect of tumor necrosis factor (TNF)- α on AZT-induced osteoclastogenesis

TNF- α is a major inflammatory cytokine and has been shown to be a stimulator of bone resorption *in vivo*.⁴² TNF- α requires the presence of RANKL to induce osteoclastogenesis, although the levels of RANKL required are quite low.¹⁷ During HIV infection the levels of TNF- α are greatly increased.^{43,44} We therefore determined whether TNF- α is involved in AZT-enhanced osteoclastogenesis. RAW264.7 cells were treated with a single dose of TNF- α at 5 ng/ml or TNF- α plus RANKL in the presence and absence of AZT. Treatment with TNF- α plus RANKL resulted in greatly enhanced TRAP intensity compared to treatment with TNF- α alone; the intensity was similar to that observed with RANKL alone (Fig. 3). Furthermore, addition of TNF- α did not affect the TRAP intensity of cells treated either with AZT alone, or with AZT plus RANKL. TNF- α alone slightly, but not significantly, increased TRAP intensity ($p > 0.05$ vs. no treated control; Fig. 3). Similar results occurred with AZT and AZT plus TNF- α . These results indicate that under the assay conditions employed here, TNF- α is not capable of enhancing the osteoclastogenic action of RANKL, it cannot substitute for RANKL in AZT-enhanced osteoclastogenesis, and cannot further enhance RANKL-dependent AZT-enhanced osteoclastogenesis.

Kinetics of AZT-induced osteoclastogenesis

Osteoclastogenesis consists of multiple stages, including cell adhesion, proliferation, differentiation, and fusion. To determine which stage is affected by AZT, RAW264.7 cells were cultured with AZT for the indicated day(s) in the presence of RANKL. For the first 2 days, addition of AZT dramatically increased osteoclastogenesis rendering TRAP intensity doubled to that generated in the cells treated with RANKL alone (Fig. 4A). Simultaneously, the expression of both TRAP and calcitonin receptor (CTR) genes was also markedly increased (Fig. 4B). After the third day, however, neither the TRAP intensity nor the expression of TRAP and CTR was further increased by AZT. These results support the concept that AZT potentially stimulates osteoclastogenesis, at least in part, at the early stages of osteoclastogenesis.

Effect of other NRTIs, alone and in combination, on osteoclastogenesis

To determine the osteoclastogenic potential of NRTIs other than AZT, the effects of ddI and 3TC on osteoclast differentiation were tested *in vitro* and *in vivo*. In the absence of RANKL, neither of these NRTIs stimulated the differentiation of RAW264.7 cells into TRAP-positive osteoclasts. The TRAP intensity was significantly greater when the RAW264.7 cells were cultured with ddI in the presence of RANKL than when cultured with RANKL alone (Fig. 5A). Similarly, the TRAP intensity was significantly greater when the RAW264.7 cells were cultured with 3TC in the presence of RANKL than when cultured with RANKL alone (Fig. 5B). These results suggest that the enhancement of RANKL-induced osteoclastogenesis is a common characteristic of NRTI drugs.

As certain combinations of NRTIs (AZT + ddI, AZT + 3TC, and AZT + ddI + 3TC) are commonly used in combination in HAART, we next determined the effects of these combinations on RANKL-induced osteoclastogenesis. None of these combinations of NRTIs enhanced osteoclastogenesis in the absence of RANKL. Although the individual NRTIs

enhanced osteoclastogenesis in the presence of RANKL, there was no evidence of additive or synergistic effects on osteoclastogenesis (Fig. 6).

To determine whether additive or synergistic effects of combined NRTI treatments occur *in vivo*, three groups of mice were fed PBS as control, AZT, or AZT plus ddI for 4 weeks. A comprehensive histological and histomorphometric analysis was performed and is then summarized in Fig. 7. The BMD and BV/TV were significantly lower in mice treated with AZT alone or AZT + ddI than in the control mice ($n = 6$, $p < 0.05$). Histomorphometry analysis of three vertebrae from each animal indicated that the parameters indicative of osteoclastogenesis, including OcN/BS and OcS/BS, were higher in the NRTI-treated mice than control mice. In keeping with the results generated using RAW264.7 cells, there was no statistical difference in the measures of osteopenia and osteoclastogenesis between the mice treated with AZT alone or AZT in combination with ddI.

Osteoblast parameters, including the number (ObN/BS) and surface (ObS/BS) of osteoblasts, as well as osteoblast perimeter, were not significantly different in the NRTI-treated mice compared to the control mice. Thus, the NRTI-induced bone loss appears to be associated with heightened osteoclastogenesis and to have little effect on osteoblastogenic bone formation.

Effect of AZT or RANKL on apoptosis

Mitochondrial toxicity induced by AZT and other NRTIs has been reported to accelerate apoptosis, which could account for the pathogenesis of many metabolic degenerative complications. To determine whether AZT accelerates apoptosis, RAW264.7 cells were cultured with or without AZT in the presence and absence of RANKL for 2 days followed by measuring apoptosis. We observed that AZT alone significantly stimulated apoptosis, whereas RANKL did not induce apoptosis of RAW264.7 cells (Fig. 8). Notably, the increased apoptosis induced by AZT was inhibited by RANKL, suggesting a protective action of RANKL from apoptosis induced by AZT.

AZT-induced osteoclastogenesis using human PBMCs

Currently, the most frequently used method for analysis of osteoclastogenesis is analysis of the generation of TRAP-positive cells using the murine preosteoclast cell line (RAW264.7) as described above. Recently, it has been reported that functional osteoclasts can be differentiated *in vitro* from human PBMC.^{45,46} To determine osteoclastogenesis using human PBMCs, human PBMCs (2.5×10^6 /well in 6-well plate) were cultured in the presence of M-CSF (25 ng/ml) and RANKL (100 ng/ml) for ~2 weeks with and without addition of AZT.^{47,48} The results generated in the absence of AZT confirmed that osteoclastogenesis can be demonstrated using human PBMC. In the presence of AZT plus RANKL, the number of TRAP-positive osteoclasts was enhanced approximately 3-fold compared to the number observed on treatment with RANKL alone, which is consistent with the effects seen using the murine RAW264.7 cell line. The morphology of the typical TRAP-positive multi-nucleated osteoclasts containing more than three nuclei is illustrated in Fig. 9A. Moreover, there was a linear correlation of osteoclastogenesis of PBMC treated with RANKL plus AZT versus RANKL alone ($r^2 = 0.82$) in the small group of six different PBMC samples tested ($n = 6$, Fig. 9B). These results indicate that the results we have generated using mouse cell lines and an *in vivo* mouse model are of relevance to human cells and provide a model in which the effects of NRTIs on osteoclastogenesis can be studied in the setting of HIV infection.

DISCUSSION

In a previous study, we have shown that AZT markedly enhances osteoclastogenesis of RAW264.7 cells and bone marrow osteoclast progenitors when cultured *in vitro* in the presence

of RANKL.⁸ In this study, we confirmed that RANKL is required for this effect as OPG blocked AZT-stimulated osteoclastogenesis in an *in vitro* assay. AZT and other NRTIs have mitochondrial toxicity accelerating apoptosis and inducing metabolic degenerative complications, such as diabetes, lipodystrophy, and heart disease. However, no effect on apoptosis was observed when osteoclastogenesis was induced by AZT in the presence of RANKL. AZT alone enhanced apoptosis, an effect countered by RANKL (Fig. 8). The molecular mechanism responsible for RANKL protecting cells from AZT-induced apoptosis has not been elucidated. It may be associated with activation of some intracellular signaling pathways, such as ERK or NF- κ B.⁴⁹ Thus, AZT-induced enhancement of osteoclastogenesis may be mediated primarily through an effect on RANK/RANKL modulated osteoclastogenesis, rather through an effect on osteoclast resorption activity or the rate of apoptosis of osteoclasts.

ALN, when added to cultures, did not inhibit RANKL-dependent AZT-enhanced osteoclastogenesis. ALN is thought to inhibit osteoclast activity and increase osteoclast apoptosis^{24–26} and it has been demonstrated that bisphosphonates containing nitrogen are capable of binding to bone surfaces and inhibiting bone resorption. The enhancement by AZT of RANKL-mediated osteoclastogenesis gradually declines in the presence of high doses of ALN. This may be associated with cytotoxicity at high doses of ALN leading to increased apoptosis. ALN has also been found to increase OPG expression, which might play a role in this *in vitro* system. ALN and other bisphosphonates are capable of directly stimulating OPG gene expression and protein secretion from human osteoblasts.^{50,51} Although ALN did not inhibit RANKL-dependent AZT osteoclastogenesis in our *in vitro* assays, it has been reported that in a group of AIDS patients on HAART who were treated with ALN for 96 weeks, 27% of the patients on ALN versus 96% the patients without ALN presented with osteoporosis. This suggests that once-weekly oral ALN may be an effective and safe treatment for HIV-associated osteoporosis.⁵² It is possible that ALN compensates indirectly for the NRTI-induced osteoclastogenesis.

The osteoclastogenic effect of AZT occurs, at least in part, during the early (days 0–2) stage of osteoclast differentiation. This stage is critical for M-CSF-dependent proliferation of osteoclast progenitors.^{53,54} The expression of osteoclastogenesis-associated genes during the early stage was accelerated in the presence of RANKL stimulation. Similar results have also been reported for the kinetics of OPG-mediated inhibition of osteoclastogenesis. OPG strongly affected the early (days 0–3) stage of osteoclast formation.^{53,54} Transforming growth factor (TGF)- β has been reported to be a strong inhibitor when present in the early stage (days 0–3) of culture but had only a weak effect when added after day 3.⁵³

It has been reported that TNF- α exerts its bone-eroding effects by directly promoting the differentiation of osteoclast precursors and increasing the expression of RANKL through induction of interleukin-1.^{15,55} HIV-infected patients have high levels of RANKL,^{56,57} which not only augments HIV replication forming a positive feedback loop,⁵⁷ but also accelerates osteoclast formation and bone loss in the presence of TNF- α .^{5,58–60} This discrepancy may be associated with the lack of a specific bone microenvironment of HIV infection in cultured RAW264.7 cells. Bone loss in HIV infection may result from pathophysiological interactions between HIV infection, HAART, osteoclasts, osteoblasts, and other elements within the bone microenvironment. Our *in vitro* studies using RAW264.7 cells indicated that TNF- α treatment alone promoted slight, but not significant osteoclastogenesis, suggesting no osteoclastogenic effect of TNF- α in the absence of RANKL. Moreover, AZT in addition to TNF- α had no effect of osteoclastogenesis.

In this study, we show that two other NRTI drugs, ddI and 3TC, are also capable of enhancing osteoclastogenesis in a RANKL-dependent manner *in vitro*, and confirmed that ddI induces

osteoclastogenesis and osteopenia *in vivo*. These data suggest that enhancement of osteoclastogenesis in a RANKL-dependent manner is a common feature of all NRTIs. They are in concordance with the evidence showing that many NRTIs can markedly reduce BMD in both humans and monkeys^{61,62} and AIDS patients treated with tenofovir (TDF) alone have been reported to have a higher incidence of osteopenia and osteoporosis than patients not receiving NRTIs.⁶³ It also has been reported that the risk of associated metabolic bone disease varies among the individual NRTIs, however, with 3TC and ABC being considered to represent NRTIs with a lower risk of bone toxicities. Our previous studies reported that the osteoclastogenic effect of AZT is associated with the dose of RANKL.⁸ Because of high levels of RANKL in HIV-infected patients, the effect of NRTIs on RANKL-mediated osteoclastogenesis may be further enhanced *in vivo*.^{56,57}

In HAART, NRTI drugs are generally combined in a strategy in which two or more NRTI drugs are used to efficiently interrupt disease progression and significantly reduce morbidity and mortality.^{64–67} We did not find, however, that osteoclastogenesis was accelerated or exacerbated when two or more NRTIs were administered *in vitro* or the *in vivo* animal model and neither additive nor synergistic effects were observed. These results concur with the report that the incidence of osteopenia and osteoporosis in antiretroviral-naïve HIV-positive patients treated with TDF alone is similar to that observed in patients treated with the combination of 3TC plus efavirenz over 144 weeks.⁶³ However, some combinations have been avoided, due to their high rates of toxicity, drug resistance, a greater risk of failure,⁶⁸ but not their high risk of producing osteopenia or other side effects.^{69,70} For example, ABC + 3TC (Epzicom), AZT + 3TC (combivir), and ABC + AZT + 3TC (Trizivir) are currently listed as preferred NRTI regimens with less toxicity, whereas AZT + d4T, ddI + d4T, and ddI + TDF are frequently avoided in AIDS therapy.⁷¹ Thus, selecting suitable NRTI drugs and their combination increasing antiretroviral and decreasing osteoclastogenic activities will be critical for AIDS therapy. NRTI drugs are frequently used with various protease inhibitors. Whether the protease inhibitors exacerbate or ameliorate osteoclastogenesis remains to be investigated. Interestingly, Wang *et al.* have reported that the protease inhibitor ritonavir blocks osteoclastogenesis, thereby decreasing bone loss.⁷²

Although bone marrow is usually used as a source of osteoclast precursors, functional osteoclasts can be differentiated *in vitro* from human PBMC,^{45,46} and this technique has been facilitated by the commercial availability of recombinant RANKL.^{45,46} Osteoclast formation from PBMC has been investigated in postmenopausal osteoporosis, psoriatic arthritis,^{73–75} and various osteoclastogenic bone disorders such as Paget's disease and multiple myeloma.^{73,76–78} We were able to confirm that AZT profoundly enhances differentiation of osteoclasts from human osteoclast precursors and does so in a RANKL-dependent manner. AIDS patients have a high incidence of low BMD, but it is difficult to obtain bone marrow biopsies in these patients for examination of the number and function of osteoclasts using traditional techniques. Thus, this *in vitro* PBMC-based osteoclastogenesis system may be extremely important in predicting low BMD and increased numbers of osteoclast number in HIV-infected individuals and patients with AIDS.

In summary, the present study determined the effects of various osteoclastogenic and antiosteoclastogenic factors on osteoclastogenesis induced by NRTIs. AZT and other NRTIs are capable of enhancing osteoclastogenesis *in vitro* and *in vivo*, but no synergistic action exists with combinations of the NRTIs. TNF- α neither substitutes for RANKL nor enhances the RANKL-dependent NRTI-induced osteoclastogenesis. OPG inhibits or ameliorates osteoclastogenesis. Peripheral osteoclast precursors from PBMCs are able to differentiate into osteoclasts in the presence of RANKL and M-CSF. Furthermore, osteoclast differentiation from osteoclast precursors in PBMC is accelerated by AZT and other NRTIs consistent with our findings using RAW264.7 cells. The *in vitro* osteoclastogenesis assay using human PBMC

represents an ideal noninvasive assay system for further analysis of the bone status in HIV-infected individuals receiving HAART.

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FIG 1.

Effect of OPG on AZT-enhanced osteoclastogenesis. RAW264.7 cells were incubated with individual AZT (25 μ M), OPG (100 ng/ml), or RANKL (50 ng/ml) and AZT plus RANKL in the absence and presence of OPG for 4 days. Osteoclastogenesis was determined by TRAP staining. TRAP intensity per well is expressed as the mean $\pm$ SE of three individual experiments. ***Statistically significant difference from OPG-treated samples ($p < 0.001$). Representative TRAP-stained plates are shown at the top.

FIG 2.

Effect of alendronate (ALN) on AZT-enhanced osteoclastogenesis. RAW264.7 cells were cultured with RANKL (50 ng/ml) alone or RANKL plus AZT (25 μ M) in the presence of indicated doses of ALN (0– 10^{-6} M) for 4 days. Osteoclastogenesis was determined by TRAP staining. Data are presented as the mean $\pm$ SEM of TRAP intensity per well of three individual experiments. *Statistical significance between RANKL (R) and RANKL plus AZT treated (R + A) at each concentration of ALN ($p < 0.05$).

FIG 3.

Effect of TNF- α on AZT-enhanced osteoclastogenesis. RAW264.7 cells were incubated with medium, AZT (25 μ M), RANKL (50 ng/ml), TNF- α (5 ng/ml) alone, and various combinations of RANKL + AZT, TNF- α + AZT, RANKL + TNF- α , and TNF- α + RANKL + AZT for 4 days. Osteoclastogenesis was determined by TRAP staining. Data are presented as the mean $\pm$ SEM of TRAP intensity per well of three individual experiments. Statistical significance of $*p < 0.05$ and $**p < 0.01$, respectively, is indicated.

FIG 4.

Effect of the stage of cell proliferation and differentiation on AZT-enhanced osteoclastogenesis. **(A)** RAW264.7 cells (5.0×10^4) were cultured with RANKL (50 ng/ml) for 3 days or with AZT (25 μ M) for indicated day(s) in the presence of RANKL. In RANKL plus AZT treatment, AZT (25 μ M) was removed from the medium on the indicated day. The treatment schedule is shown on the left. Osteoclastogenesis was determined by TRAP staining. Data are presented as the mean $\pm$ SEM of TRAP intensity per well of three individual experiments. Statistical significance (** $p < 0.01$ and *** $p < 0.001$) compared to RANKL treatment alone is indicated. **(B)** RAW264.7 cells were cultured with RANKL (R, 50 ng/ml) or RANKL plus AZT (R + A) for 1, 2, and 3 days. Total mRNAs were isolated as per the manufacturer's instruction. RT-PCR for TRAP, CTR, RANK, and GAPDH genes was performed as described in Materials and Methods.

FIG 5.

Effect of NRTIs on the osteoclastogenesis of RAW264.7 cells. RAW264.7 cells were exposed to individual NRTIs, ddI, and 3TC, at the indicated concentrations in the absence and presence of RANKL (50 μ g/ml) for 4 days. Osteoclasts were detected by TRAP staining. Data are presented as the mean $\pm$ SEM of TRAP intensity per well of three individual experiments. Statistical significance between RANKL and RANKL plus ddI- or 3TC-treated samples is indicated: * p < 0.05 and ** p < 0.01.

FIG 6.

Effect of combinations of NRTIs on osteoclastogenesis. RAW264.7 cells were cultured with AZT (10 μ M), ddI (2 μ M), or 3TC (5 μ M), and indicated combinations of two or three NRTIs in the presence of RANKL (50 ng/ml) for 4 days. RANKL alone without NRTIs was also performed. TRAP staining was performed to determine osteoclastogenesis. Data are presented as the mean $\pm$ SEM of TRAP intensity per well of three individual experiments. Statistical significance between RANKL- and RANKL plus NRTI-treated samples is indicated: * p < 0.05 and ** p < 0.01.

FIG 7.

Bone mineral density and histomorphometric analysis. Mice, 5 months of age, were orally fed PBS (control), AZT alone (AZT), or AZT plus ddI (AZT + ddI) per day for 30 days. **(A)** Bone mineral density (BMD) was measured by DEXA (* $p < 0.05$ in a one-way ANOVA compared to controls). Vertebrae were fixed, decalcified, and stained with trichrome.

Histomorphometry was analyzed in three vertebrae for each animal. **(B)** Ratios of osteoclast surface (gray bar, OcS) or osteoclast number (open bar, OcN) to bone surface (BS) are shown. The results are presented as the mean $\pm$ SEM and p value in the one-way ANOVA compared to controls; * $p < 0.05$ and *** $p < 0.001$ compared to control. **(C)** Ratios of trabecular volume to tissue volume (black bar BV/TV) and osteoblast surface (gray bar, ObS) and osteoclast

number (open bar, OcN) to bone surface (BS) are shown. $*p < 0.05$ in the one-way ANOVA compared to controls.

FIG 8.

Effect of AZT on apoptosis in RAW264.7 cells. RAW264.7 cells were cultured with AZT in the presence and absence of RANKL (R). After 2 days, cells were harvested and double-stained with Annexin V and PI and then apoptotic and necrotic cells were detected by flow cytometry. Values are mean $\pm$ SE obtained from three samples (* $p < 0.05$ compared to untreated control RAW264.7 cells).

FIG 9.

Osteoclastogenesis induced by AZT in human PBMC. Fresh human PBMCs were prepared and cultured overnight and then attached macrophages were isolated (5×10^5). Macrophages were cultured with M-CSF (25 ng/ml) and RANKL (100 ng/ml) in the presence of indicated treatments for 14 days. **(A)** Mature osteoclasts were identified by their morphology and TRAP staining. **(B)** TRAP intensity per well for six preparations of isolated PBMCs (mean $\pm$ SEM). **(C)** Correlation of osteoclastogenesis from fresh PBMC treated with RANKL plus AZT versus RANKL alone ($r^2 = 0.82$).