Select HIV Protease Inhibitors Alter Bone and Fat Metabolism ex Vivo*

Human immunodeficiency virus (HIV) therapies have been associated with alterations in fat metabolism and bone mineral density. This study examined the effects of HIV protease inhibitors (PIs) on bone resorption, bone formation, and adipocyte differentiation using *ex vivo* cultured osteoclasts, osteoblasts, and adipocytes, respectively. Osteoclast activity, measured using a rat neonatal calvaria assay, increased in the presence of nelfinavir (NFV; 47.2%, \( p = 0.001 \)), indinavir (34.6%, \( p = 0.001 \)), saquinavir (24.5%, \( p = 0.001 \)), or ritonavir (18%, \( p < 0.01 \)). In contrast, lopinavir (LPV) and amprenavir did not increase osteoclast activity. In human mesenchymal stem cells (hMSCs), the PIs LPV and NFV decreased osteoblast alkaline phosphatase enzyme activity and gene expression significantly (\( p < 0.05 \)). LPV and NFV diminished calcium deposition and osteoprotegrin expression (\( p < 0.05 \)), whereas the other PIs investigated did not. Apopidogenesis of hMSCs was strongly inhibited by saquinavir and NFV (>50%, \( p < 0.001 \)) and moderately inhibited by ritonavir and LPV (>40%, \( p < 0.01 \)). Expression of diacylglycerol transferase, a marker of adipocyte differentiation, decreased in hMSCs treated with NFV. Amprenavir and indinavir did not affect adipogenesis or lipolysis. These results suggest that bone and fat formation in hMSCs of bone marrow may be coordinate down-regulated by some but not all PIs.

Highly active antiretroviral therapy (HAART)\(^*\) is a therapeutical approach for HIV infection that involves combined treatment with three classes of anti-HIV drugs: protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors, and nucleoside reverse transcriptase inhibitors (NRTIs; Ref. 1). Over the last few years a number of unusual adverse events have been observed when HAART is used as a long term therapy (2). Some common complications associated with HAART are: adverse events related to the use of NRTIs (e.g. neuropathy, myopathy, pancreatitis, and lactic acidosis; Ref. 3), metabolic alterations or lipodystrophy (fat redistribution, insulin resistance, and dyslipidemia; Ref. 2), and bone disorders (osteonecrosis and osteoporosis; Refs. 4–7). Osteonecrosis has been documented in case reports of HIV patients; however, some of these reports predate HAART, and there is no firm evidence that osteonecrosis is associated with HAART (8, 9). Many factors may influence bone and fat metabolism and could lead to bone dysfunction in HIV patients including the presence of viral infection, therapeutic drugs (PIs, NRTIs, non-nucleoside reverse transcriptase inhibitors, or combination therapies), cellular response to the virus/drug, and immune function in the affected individual.

Decreased bone formative and increased bone resorptive serum markers have been observed in subjects receiving HAART (4, 7). However, there are conflicting reports on the cause of the bone disorders observed in these patients. Carr et al. (6) have reported a connection between low pre-therapy body weight, asymptomatic lactic acidemia, and osteopenia in HAART patients. Regardless of specific HIV drug treatment, Huang et al. (7) reported a lower bone mineral density (BMD) associated with an increase in abdominal visceral fat. Interestingly a longitudinal study report (10) demonstrates that there may be an increase or no change in BMD after treatment with certain PIs. Contrary to the above reports, Tebas et al. (5) found no link between increased lipodystrophy and lower BMD in their cross-sectional studies. Instead they suggest a link in the development of osteopenia and osteoporosis to the PIs received by HAART patients, although the role of current or previous NRTI use on bone mineral density was not addressed (11).

These studies reveal that the effect of HAART on alterations in BMD remains unclear. This is probably due to the complexity of HAART, which can involve a treatment choice of up to 16 drugs in various combinations. Furthermore the response to HAART can be influenced by pre-therapy body weight, progression of the viral infection, and its effect on bone metabolism (12). In addition, individual patients may have genetic traits or be exposed to environmental factors that influence their response to HIV and to HAART and alter their risk for the development of osteoporosis.

Osteoblasts (OBs) and osteoclasts (OCs) are derived from different cell lineages and play important roles in bone metabolism. OBs are derived from stromal cells or mesenchymal stem cells (hMSCs) within the bone marrow. OCs are derived from hematopoietic cells and are distantly related to monocytes and macrophages. OBs are involved in active bone formation while OCs are involved in bone degradation and resorption. The functions of OBs and OCs balance one another during normal bone metabolism and any alterations in the function or formation of either of these cell types may result in the development of osteopenia or osteoporosis. The effect of individual HIV-PIs on isolated OB and OC cells remains unknown.

PIs have proven to be a very effective drug class for the control of HIV infection by inhibiting the aspartyl HIV protease and interfering with formation of mature viral particles. It is important to note that these drugs vary structurally, and

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\(^{2}\) These abbreviations are used: HAART, highly active antiretroviral therapy; HIV, human immunodeficiency virus; PI, protease inhibitor; NFV, nelfinavir; IDV, indinavir; SQV, saquinavir; RTV, ritonavir; LPV, lopinavir; APV, amprenavir; hMSC, human mesenchymal stem cell; AIP, alkaline phosphatase; NRTI, nucleoside reverse transcriptase inhibitor; BMD, bone mineral density; OB, osteoblast; OC, osteoclast; OS, osteogenic stimulation; PTH, parathyroid hormone; RT, real time; OPG, osteoprotegrin; OPGL, osteoprotegrin ligand; DGAT, diacylglycerol acyltransferase; LPL, lipoprotein lipase.
mechanistic studies are essential to determine intercellular variability in the development of adverse events (such as osteoporosis). This study uses hMSCs and rat calvaria to study how PIs may influence bone and fat metabolism ex vivo. All commercially available PIs were individually studied in these HIV-free systems. To elucidate and differentiate the individual effect of each PI on bone, specific measurements of osteoclast activity/bone resorption (measured in rodent calvaria), bone formation, adipocyte formation, and fat degradation in the presence of physiological PI concentrations were examined.

MATERIALS AND METHODS

Dexamethasone, sodium β-glycerophosphate, Me₂SO, ascorbic acid 2-phosphate, isobutylmethylxanthine, alkaline phosphatase (ALP) diagnostic kit no. 86, and calcium diagnostic kit no. 587 were purchased from Sigma. The PIs used in these studies, amprenavir (APV), indinavir (IDV), lopinavir (LPV), nefinavir (NFV), ritonavir (RTV), and saquinavir (SQV), were obtained from GlaxoSmithKline Inc. chemical stores. Since PIs are insoluble at >40 μM in aqueous solutions, Me₂SO was used to make concentrated stock solutions as reported previously (13). We have observed (13) that less than 0.1% Me₂SO has no effect on cell growth and differentiation and hence is a satisfactory vehicle and contaminant. Human osteoblastic cell lines were purchased from BioWhittaker Inc. (Walkersville, MD). The measurements for osteoblastic and adipogenic parameters were conducted on differentiated cells that were no longer proliferating. Cell culture and lipid accumulation assays were done following published procedures (14, 15). In summary, hMSCs (passages 3–5) were plated at a density of 10⁴ cells/cm² and cultured in Dulbecco’s minimum essential medium containing 10% fetal bovine serum purchased from BioWhittaker Inc.

Osteogenic differentiation was induced using 0.1 μM dexamethasone, 0.05 mM ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (osteogenic stimulation (OS) medium) within 24 h of plating. Simultaneously various concentrations of PIs were added to the cells, which were incubated with 10 μM PI for 48 h, and the calcium release was quantified. The data are expressed as the percentage of calcium released relative to vehicle-treated cells using PTH as a positive control. The mean and S.D. are representative data (each point determined in triplicate) for two or more independent experiments. C, OPG gene expression was measured by real time PCR (see “Materials and Methods”) on Day 16 of hMSC osteogenic differentiation in the presence of 5 μM NFV or a 10 μM concentration of all other PIs. For each treatment the different DNA samples were isolated, and each one was analyzed in duplicate during the real time PCR. Results from each group were averaged and compared with untreated cells to provide a p value using the Student’s t test. Significant differences (p < 0.05) between control (Me₂SO) and treated groups are denoted by *. In all experiments, the solvent used for the PIs, 0.1% Me₂SO, was included as a control. DMSO, Me₂SO.

RESULTS AND DISCUSSION

OC activity was measured using a rat neonatal calvaria assay that monitors calcium release as a measure of bone resorption. The effect of a 10 μM concentration of the six PIs (APV, IDV, LPV, NFV, RTV, and SQV) on OC activity was examined. Recombinant PTH1 was used as a positive control. OC activity was calculated and expressed as the percentage of calcium released. Fig. 1A shows that OC activity increased in the presence of NFV (47.2%, p < 0.01), IDV (34.6%, p < 0.01), SQV (24.3%, p < 0.01), and RTV (18%, p < 0.01). APV and LPV did not alter OC activity significantly (p > 0.05), indicating that these two drugs do not alter bone resorption in ex vivo experiments. hMSCs isolated from marrow aspirates have the potential to differentiate into several mesenchymal tissues, including bone, cartilage, adipose, tendon, muscle, and marrow stroma (Ref. 15; i.e. adipocytic, chondrocytic, and osteogenic lineages). After exposure to OSM medium, hMSCs deposit a calcium-enriched matrix after 15 days. This deposit is readily measured using a colorimetric calcium assay (18). In the absence of OSM, hMSCs do not deposit detectable amounts of calcium during cell culture. hMSCs were exposed to 10–20 μM PIs in OSM medium, and mineralization of the extracellular matrix was measured (Fig. 1B). Calcium accumulation was inhibited in hMSCs treated with 10 μM NFV (37.6%, p = 0.016) and LPV (20.89%, p = 0.057; see Fig. 1B). Other PIs did not alter calcium accumulation significantly (p > 0.05). These results indicate that some PIs accelerate bone resorption by increasing OC activity (NFV, SQV, IDV, and RTV), and some PIs inhibit bone formation and calcium deposition (NFV and LPV) by decreasing OB activity.

Osteoprotegrin ligand (OPGL) binds and activates the receptor-activated NF-κB on the surface of the OC (19). Osteoprotegrin (OPG) is a member of the soluble tumor necrosis factor receptor 1 family expressed by the OB and is a soluble decoy

2 R. G. Jain and J. M. Lenhard, unpublished data.
receptor for OPGL. OPG sequesters OPGL, thereby preventing binding to receptor-activated NF-κB, OC activity, and bone resorption. Decreases in OPG expression by an osteoblast will lead to an increase in osteoclast activity due to the increased availability and hence binding of OPG to the osteoclast. Gene expression of OPG was measured in hMSCs induced to differentiate into OBs for 16 days in the presence of 5–10 μM PIs (Fig. 1C). Treatment with 5 μM NFV decreased OPG expression by ~50% (p = 0.04) compared with Me2SO-treated control cells. LPV (10 μM) also decreased OPG expression significantly (33%, p = 0.03), whereas the other PIs did not (p > 0.05). These results indicate that some PIs (NFV and LPV) alter expression of OPG and bone formation/resorption pathways, while other PIs do not alter these pathways _ex vivo_. One hypothesis is that the decreased osteogenesis and OPG expression could lead to increased osteoclastogenesis and bone resorption potentially explaining an underlying mechanism associated with NFV treatment. However, these data also indicate that not all PIs activate the OPG/OPGL receptor pathway in osteoblasts, and there may be other unknown mechanisms by which these PIs (e.g. RTV, IDV, and SQV) stimulate osteoclast activity in the rat calvaria assay.

**Select PIs Inhibit Osteogenesis in hMSCs—**To further assess the effects of HIV PIs on osteogenic differentiation, hMSCs were cultured in the presence of various PIs under conditions permissive for osteogenesis (15). Osteogenic differentiation is associated with increased ALP activity, calcium accumulation, increased expression of osteogenic genes, and morphological change (spindle shape becomes cuboidal; Refs. 14 and 18). Exposure to OS medium for 8 days results in a significant increase in ALP activity, and ALP activity continues to increase linearly for the next 8 days (14, 18). ALP activity was measured on Day 14 after hMSCs were treated with 10 μM HIV PIs in OS medium (control cells were treated with Me2SO). ALP activity was significantly inhibited (p < 0.01) in the presence of NFV (>63%), SQV (53%), LPV (48.8%), and RTV (29.5%, p < 0.05, Fig. 2A). Other PIs did not alter ALP activity significantly (APV and IDV by 20%, p > 0.05), indicating that PIs have pharmacologically distinct effects on osteogenic differentiation _ex vivo_. ALP gene expression was examined in differentiated hMSCs on Day 16 after exposure to PIs in OS medium. ALP gene transcription decreased significantly in the presence of LPV, NFV, and RTV (p < 0.03); a smaller decrease in ALP transcription was observed in IDV- and APV-treated cells (p > 0.05) (Fig. 2B). Upon microscopic examination, there was a 10–50% reduction in the total number of cells in the NFV- (and to a certain degree LPV-) but not the other PI-treated cells in addition to the decrease in the total number of cells stained positive for ALP enzyme activity as visualized by light microscopy (Fig. 2C). Likewise NFV has demonstrated cellular toxicity under some _in vitro_ conditions as reported by Dowell _et al_. (20).

**Adipogenic Differentiation of hMSCs in the Presence of PIs—**A number of _in vitro_ studies have examined the effects of PIs on murine (13, 20) and human adipocyte differentiation (21). These studies indicate that different PIs have different effects on adipocyte function and differentiation. In this study, we utilized the inherent properties of a select population of bone marrow cells, the hMSCs, to study the effect of PIs on adipocyte formation and degradation.

The effects of PIs on hMSC adipocyte differentiation were examined in the presence of 10–20 μM PIs. Adipogenic differentiation was assessed on Day 11 by measuring total lipid accumulation (Fig. 3A). Total lipid accumulation was significantly reduced in the presence of 20 μM SQV (59.2%, p < 0.001) and NFV (51.6%, p < 0.001) and moderately by LPV (48.8%, p < 0.01) and RTV (44%, p < 0.01). We previously demonstrated that select PIs stimulate lipolysis in murine adipocytes (13). Lipolysis was also measured in fully differentiated hMSC adipocytes exposed to PIs for 18–24 h (data not shown) at multiple doses. Free fatty acids were released in a dose-dependent manner in cells treated with >20 μM NFV. No other PIs increased lipolysis, indicating that different PIs alter fat metabolism within the bone marrow through different mechanisms.

Adipocyte differentiation involves changes in expression of certain genes essential for lipid metabolism. Therefore, RT-PCR was used to measure expression of diacylglycerol acyltransferase (DGAT) and lipoprotein lipase (LPL) in hMSCs differentiating into adipocytes that were exposed to PIs. DGAT expression decreased in the presence of 5 μM NFV, a 10 μM concentration of the other PIs, or 0.1% Me2SO for 21 days. Cells were stained for ALP activity. Significant differences (p < 0.05) between control (Me2SO) and treated groups are denoted by *. In all experiments, the solvent used for the PIs, 0.1% Me2SO, was included as a control. DMEM, Me2SO.

**FIG. 2. Select protease inhibitors inhibit ALP activity.** hMSCs were differentiated into osteoblasts in the presence of 5 μM NFV or a 10 μM concentration of all other PIs. A, ALP enzyme activity was measured on Day 14 of differentiation. The means and S.D. are representative data (each point determined in triplicate) for two or more independent experiments. B, bone-specific ALP gene expression was examined on Day 16 of differentiation. ALP transcription is expressed as a percentage of vehicle-treated hMSCs. For each treatment three different RNA samples were isolated, and each one was analyzed in duplicate during the real time PCR. Results from each group were averaged and compared with untreated cells to provide a p value using the Student’s _t_ test. C, light micrographs of hMSCs grown with osteogenic supplements in the presence of 5 μM NFV, a 10 μM concentration of the other PIs, or 0.1% Me2SO for 21 days. Cells were stained for ALP activity. Significant differences (p < 0.05) between control (Me2SO) and treated groups are denoted by *. In all experiments, the solvent used for the PIs, 0.1% Me2SO, was included as a control. DMEM, Me2SO.
body bone mineral content by 0.04 n containing regimen (34). In a prospective study on subjects receiving an APV-con
14 n/H11005n BMD in HIV-infected patients treated with NFV (14 of differentiation in the presence of 5
20/H9262n experiments.

A

B

Fig. 3. Effects of PIs on adipogenesis. hMSCs were differentiated into adipocytes with an adipogenic mixture in the presence PIs. A, triglyceride accumulation was measured on Day 11 of differentiation in the presence of 20 μM PIs. The means and S.D. are representative data (each point determined in triplicate) for two or more independent experiments. B, expression of adipogenic markers was measured on Day 14 of differentiation in the presence of 5 μM NFV and a 10 μM concentration of all other PIs. The vehicle control was 0.1% Me2SO. DGAT expression was determined by Tagman/RT-PCR. For each treatment three different RNA samples were isolated, and each one was analyzed in duplicate during the real time PCR. Results from each group were averaged and compared with untreated cells to provide a p value using the Student’s t test. Significant differences (p < 0.05) between control (Me2SO) and treated groups are denoted by *. In all experiments, the solvent used for the PIs, 0.1% Me2SO, was included as a control. DMSO, Me2SO.

hMSC osteoblasts, and decreased adipogenesis; however, it did not alter rodent calvaria OC activity. In the case of IDV, OC activity increased significantly, but no change was observed in bone formation and resorption, respectively. Interestingly, NFV had properties similar to both LPV and IDV. NFV significantly increased osteoclast activity in the calvaria assay and decreased calcium accumulation and OPG expression in the hMSC osteoblasts. Taken together, these data show that individual PIs have distinct effects on bone and fat metabolism, and the structural differences between PIs could help explain the variability observed in clinical reports. Currently limited clinical data are available on BMD measurements on select PI-containing HAART regimens. In contrast to Tebas et al. (5), Nolan et al. (10) have reported that there are no alterations in BMD in HIV-infected patients treated with NFV (n = 20) and a modest increase in BMD with IDV-containing therapy (n = 34). In a prospective study on subjects receiving an APV-containing regimen (n = 14) there was an increase in the total body bone mineral content by 0.04 ± 0.01 kg (p = 0.02) over 48 weeks (22). Hence, these data support the idea that clinical studies are needed that discriminate between the effects of individual PIs and discern whether adverse events should be grouped together as a class effect (11).

As the studies reported here are limited to ex vivo conditions, further studies in vivo are needed to deduce the mechanism(s) of PI action in human subjects. While our results suggest a possible mechanism by which some PIs alter bone and fat metabolism, the data do not account for many factors including active metabolites, pharmacokinetic parameters, environmental factors, and genetic predisposition, which may influence the development of osteoporosis in the clinic. Additionally these ex vivo treatments do not account for the effect of serum protein binding and drug-drug interaction on the activities of the PIs within the patient. There are reports of NRTIs (for review, see Refs. 2 and 3) and combination therapy of PIs + NRTIs also influencing fat metabolism and mitochondrial toxicity indicating additional mechanistic studies using the NRTIs are needed.

The use of anti-HIV drugs should be evaluated based on their therapeutic benefits and potential adverse effects. Each PI used in anti-HIV treatment needs to be assessed for its specific effects with respect to lipodystrophy, osteoporosis, hyperlipidemia, and other conditions. A clinician might consider a patient’s treatment history, risk factors, and quality of life before determining the best therapy for that patient. These results indicate that certain PIs may have a minimal effect on osteoblast and osteoclast activity and could aid in the development of safer anti-HIV drugs.

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