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

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Running Title: HAART alters bone metabolism

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Abstract:

HIV therapies have been associated with alterations in fat metabolism and bone mineral density. This study examines 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 (IDV; 34.6%; p=0.001), saquinavir (SQV; 24.3%; p=0.001) or ritonavir (RTV;18%; p<0.01). In contrast, lopinavir (LPV) and amprenavir (APV) did not increase osteoclast activity. In human mesenchymal stem cells (hMSCs), the PIs LPV and NFV decreased osteoblast alkaline phosphatase (ALP) enzyme activity and gene expression significantly (P<0.05). LPV and NFV diminished calcium deposition (P<0.05), whereas the other PIs investigated did not. Adipogenesis of hMSCs was strongly inhibited by SQV and NFV (>50%; p<0.001) and moderately inhibited by RTV and LPV (>40%; p<0.01). Expression of diacylglycerol transferase, a marker of adipocyte differentiation, decreased in hMSCs treated with NFV treatment. APV and IDV did not affect adipogenesis or lipolysis. These results suggest that bone and fat formation in hMSCs of bone marrow, may be coordinately down regulated by some, but not all PIs.

Introduction:

Highly active antiretroviral therapy (HAART) is a therapeutic approach for HIV infection that involves combined treatment with three classes of anti-HIV drugs: protease inhibitors (PIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs) and nucleoside reverse transcriptase inhibitors (NRTIs; 1). Over the last few years a number of unusual adverse events have been observed when HAART is employed as a long term therapy (2). The three most common complications associated with HAART are: adverse events related to the use of NRTIs (*e.g.*, neuropathy, myopathy, pancreatitis, lactic acidosis (3), metabolic alterations or lipodystrophy (fat redistribution, insulin resistance, dyslipidemia; 2) and bone disorders (osteonecrosis and osteoporosis; 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, NNRTIs, 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) report of a connection between low pre-therapy body weight, asymptomatic lactic acidemia and osteopenia in HAART patients (6). Regardless of specific HIV drug treatment, Huang et al reported a lower bone mineral density (BMD) associated with an increase in abdominal visceral fat (7). 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) do not find a 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 therapy, which can involve 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 (hMSC) within the bone marrow. OCs are derived from hematopoetic 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 mechanistic studies are essential in order to determine interclass 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 each individual PI's effect 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, DMSO, ascorbic acid-2 phosphate, iso-butyl methyl xanthine, alkaline phosphatase (ALP) diagnostic kit #86, and calcium diagnostic kit # 587 were purchased from Sigma Chemical Co (St. Louis, MO). The PIs used in these studies, amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV) were obtained from GlaxoSmithKline Inc. chemical stores. Since PIs are insoluble at >40 μM in aqueous solutions, DMSO was used to make concentrated stock solutions as previously reported (13). We have observed (13) and unpublished data) that less than 0.1% DMSO has no effect on cell growth and differentiation and hence is a satisfactory vehicle and control for these experiments. Human mesenchymal stem cells were purchased from Biowhittaker Inc (Walkersville, MD). The measurements for osteoblastic and adipogenic parameters were conducted on differentiated cells which 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 10^3cells/cm^2 and cultured in Dulbecco's Minimum Essential Media containing 10% fetal bovine serum purchased from Biowhittaker Inc (Walkersville, MD). Osteogenic differentiation was induced using 0.1 μM dexamethasone, 0.05 mM ascorbic acid –2-phosphate and 10 mM β-glycerophosphate (osteogenic stimulation; OS) media) within 24 hours of plating.

Simultaneously, various concentrations of PIs were added to the cells which were almost >90% confluent. Measurements of ALP activity and histochemical staining in hMSC (Biowhittaker, Inc, Walkersville, MD) were performed as described (14). After 7-21 days ALP activity and histochemical staining was measured using the Sigma-Fast para-nitrophenyl phosphate substrate and the ALP-Leukocyte staining, respectively, according to the manufacturer's specifications (Sigma, St. Louis, MO). For calcium accumulation measurements, the Sigma Diagnostic Kit # 587 was used following the manufacturer's instructions. Cells were incubated in 0.6 N HCl for 24 hours and an aliquot diluted 20X into the calcium-working reagent and shaken for 3 minutes followed by an OD measurement at 575 nM. Alternatively, adipogenesis was induced by treating confluent cells for 14-21 days with 0.01 mg/ml insulin; 1 μM BRL49653, 1 μM dexamethasone, 0.5 mM IBMX, and 1 μM LG100268 for a period of three days followed by the removal of dexamethasone and IBMX for a period of 2 days. After 14-21 days in culture, cellular lipid content was measured using the Sigma Diagnostic Glycerol-Triglyceride assay (*i.e.,* Trinder reagent 337; Sigma, St. Louis, MO).

Neonatal rat calvaria were surgically removed from pregnant Wistar female rats and placed in basal media as described in Vargas et al (16). After a 24 hour stabilization period, the calvaria were incubated in basal media containing 10 μM PI for a period of 48 hours. After the incubation, the media was collected and an aliquot analyzed for calcium release using the Sigma Diagnostic Kit # 588 following the manufacturer's instructions. All assays were run in triplicate and results from each group were compared with untreated calvaria and parathyroid hormone (PTH) treated calvaria.

Total RNA was isolated using Qiagen RNeasy kits (Qiagen Inc., Valencia, CA) and quantified with Ribogreen (Molecular Probes, Eugene, OR). Taqman probes and primers were

designed to match GenBank sequences for human diacylglycerol transferase, alkaline phosphatase and lipoprotein lipase. Real time polymerase chain reactions (RT-PCR) were performed as described in (17). All samples were assayed in duplicate with three samples per group. Results from each group were averaged and compared with untreated cells to provide a *P* value using the students T test.

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 10 μM of the six PIs on OC activity was examined: amprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV) and saquinavir (SQV). Recombinant PTH was used as a positive control. OC activity was calculated and expressed as the percentage of calcium released. Figure 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 (15) i.e., adipocytic, chondrocytic and osteogenic lineages). After exposure to osteogenic stimulation (OS) media, hMSCs deposit a calcium-enriched matrix after 15 days. This deposit is readily measured using a sensitive colorimetric calcium assay (18). In the absence of OS, hMSCs do not deposit detectable amounts of calcium during cell culture. hMSCs were exposed to 10-20 μM PIs in OS media, and mineralization of the extracellular matrix was measured (Figure 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 Figure 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, SQV & LPV) by decreasing OB activity.

Osteoprotegrin ligand (OPGL) binds and activates the receptor activated NF-κB (RANK) 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 receptor for OPGL. OPG sequesters OPGL, thereby preventing binding to RANK, 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 OPGL 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 (Figure 1C). Treatment with 5 μM NFV decreased OPG expression by approximately 50% (p=0.04) compared to DMSO 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 hMSC cells

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 alkaline phosphatase (ALP) activity, calcium accumulation, increased expression of osteogenic genes and morphological change (spindle shape becomes cuboidal; 14,18). Exposure to OS media for 8 days resulted in a significant increase in ALP activity, and ALP activity continued 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 media (control cells were treated with DMSO). ALP activity was significantly inhibited ($p<0.01$) in the presence of NFV (>63%), SQV (53%), LPV (48.8%) and RTV (29.5%; $p<0.01$; Figure 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 media. ALP gene transcription decreased significantly in the presence of LPV, NFV, and RTV; a smaller decrease in ALP transcription was observed in APV-treated cells (Figure 2B). Upon microscopic examination, there was a 10-50% reduction in the total number of cells in the NFV (and to certain degree LPV) but not the other PIs treated cells, in addition to the decrease in the total number of cells stained positive for ALP enzyme activity as visualized by light microscopy (Figure 2C). Likewise, NFV demonstrated cellular toxicity under some *in vitro* conditions reported by Dowell et al. (20).

Adipogenic Differentiation of hMSC cells 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 (Figure 3A). Total lipid accumulation was significantly reduced in the presence of 20μM SQV (59.2%; $p<0.001$); NFV (51.6%; $p<0.001$) and moderately by LPV (48.8%; $p<0.01$) and RTV (44%; $p<0.01$). We previously demonstrated select PIs stimulate lipolysis in murine adipocytes (13). Lipolysis was also measured in fully differentiated hMSC adipocytes exposed to PIs for 18-24 hours (data not shown) at acute 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 (Figure 3B), whereas the other PIs did not have a significant effect. NFV caused a similar decrease in expression of LPL (data not shown). This is consistent with our previous observation that NFV decreases LPL expression in differentiating murine adipocytes (13). Taken together, these observations suggest that the loss of fat in subjects receiving HAART may, in part, be due to decreased expression of genes involved in lipogenesis and increased lipolysis.

In summary, this study demonstrates that select PIs alter OC, OB and adipocyte activity and differentiation *ex vivo*. The observation that LPV, NFV and SQV inhibit both osteogenesis

and adipogenesis in hMSCs, suggests that bone and fat metabolism may be coordinately down regulated in the bone marrow, potentially contributing to altered bone mineral density. Direct effects of PIs on OCs may contribute also to the altered bone mineral density associated with HAART. Not all PIs exhibited the same effects *ex vivo*, indicating that different PIs have distinct effects on bone and fat metabolism. For example, LPV decreased OPG expression, inhibited calcium accumulation by 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 OB ALP-activity, hMSC calcium accumulation, or adipogenesis. This suggests that LPV and IDV may have selective effects on 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 is available on BMD measurements on select PI containing HAART regimens. In contrast to Tebas et al. (5), Nolan et al (10) report of no alterations in BMD in HIV infected patients treated with NFV (n=20) and a modest increase in BMD with IDV (n=34) containing therapy. In a prospective study on subjects receiving an APV containing regimen (n=14) there was an increase in the BMD by 0.04 ± 0.01 kg (p=0.01) 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 if 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 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.

Figure Legends

Figure 1. Select Protease inhibitors increase bone resorption *ex vivo*. **A**. Measurement of osteoclast activity. Rat neonatal calvaria 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 parathyroid hormone (PTH) included as a positive control. The means and standard deviations are representative data (each point determined in triplicate) for two or more independent experiments **B**. hMSC cells were differentiated into osteoblasts in the presence of 10 and 20 μM PIs for 23 days. Calcium accumulation was measured as described in materials and methods. The means and standard deviations are representative data (each point determined in triplicate) for two or more independent experiments **C**. Osteoprotegrin (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 10 μM of all other PIs. For each treatment three different RNA samples were isolated and each one analysed 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 students T test.. Significant differences between control (DMSO) and treated groups are denoted by *P<0.05. In all experiments, the solvent used for the PIs, 0.1% DMSO was included as a control.

Figure 2. Select protease inhibitors inhibit alkaline phosphatase (ALP) activity. hMSC cells were differentiated into osteoblasts in the presence of 5 μM NFV or 10 uM of all other PIs. A. ALP enzyme activity was measured on day 14 of differentiation. The means and standard deviations 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 % of vehicle treated hMSC cells. For each

treatment three different RNA samples were isolated and each one analysed 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 students T test. C. Light micrographs of hMSC cells grown with osteogenic supplements in the presence of 5 μM NFV or 10 μM other PIs or 0.1% DMSO for 21 days. Cells were stained for ALP activity. Significant differences between control (DMSO) and treated groups are denoted by *P<0.05. In all experiments, the solvent used for the PIs, 0.1% DMSO was included as a control.

Figure 3. Effects of PIs on adipogenesis. hMSC cells were differentiated into adipocytes with an adipogenic cocktail in the presence PIs. A. Triglyceride accumulation was measured on Day 11 of differentiation in presence of 20 μM PIs. The means and standard deviations 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 10 μM all other PIs. The vehicle control was 0.1% DMSO. DGAT expression was determined by Taqman/RT-PCR. For each treatment three different RNA samples were isolated and each one analysed 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 students T test. Significant differences between control (DMSO) and treated groups are denoted by *P<0.05. In all experiments, the solvent used for the PIs, 0.1% DMSO was included as a control.

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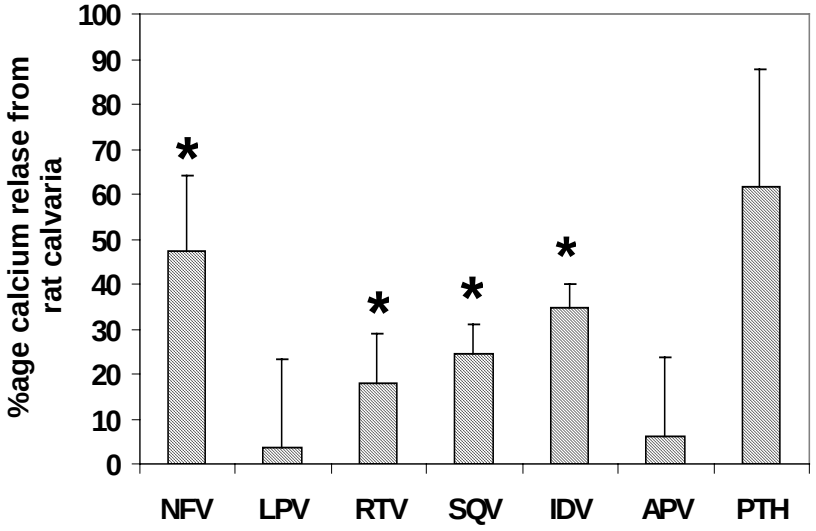
Figure 1A.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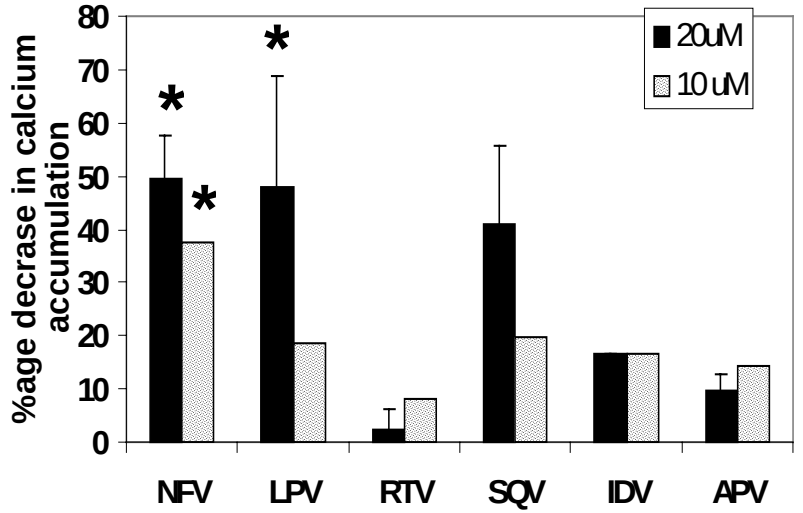
B.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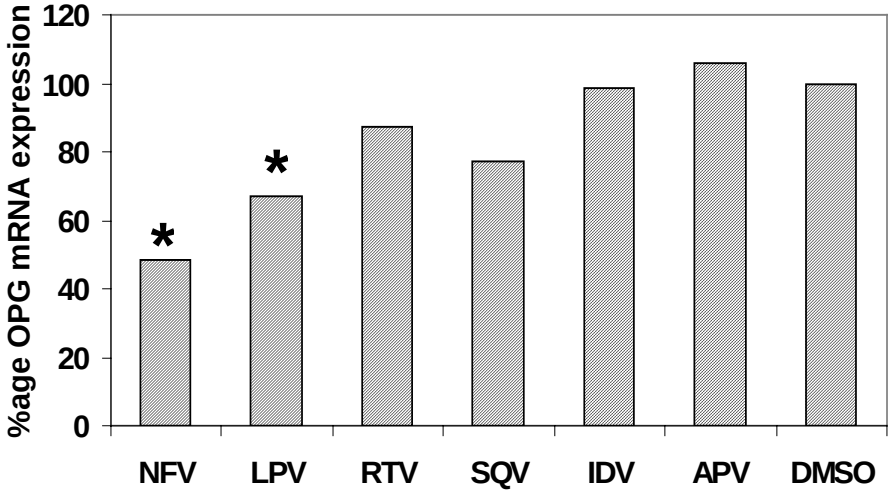
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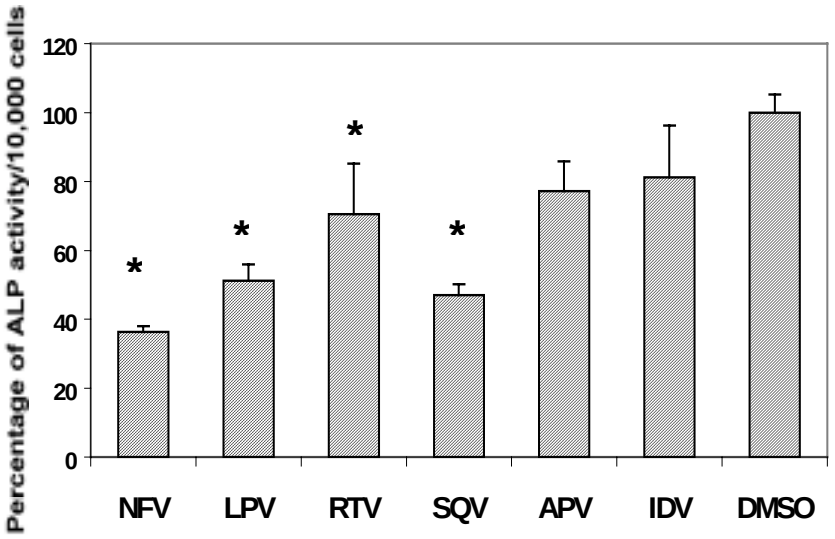
Figure 2A.


Percentage of ALP activity/10,000 cells
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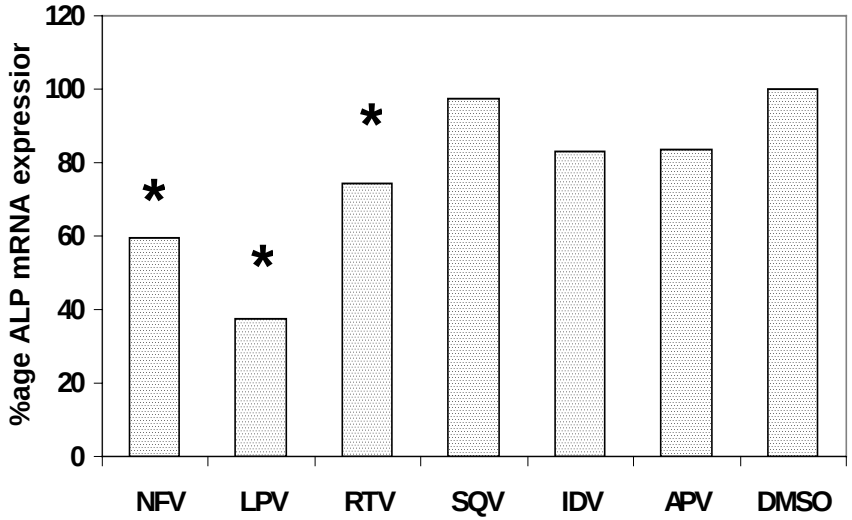
B.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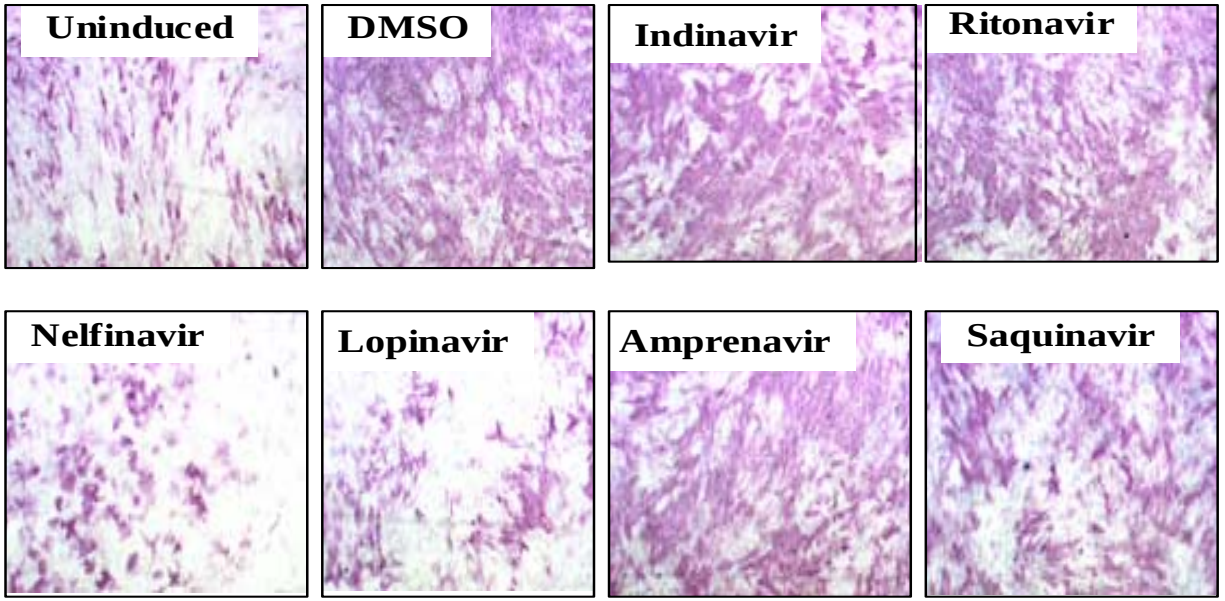
Uninduced
DMSO
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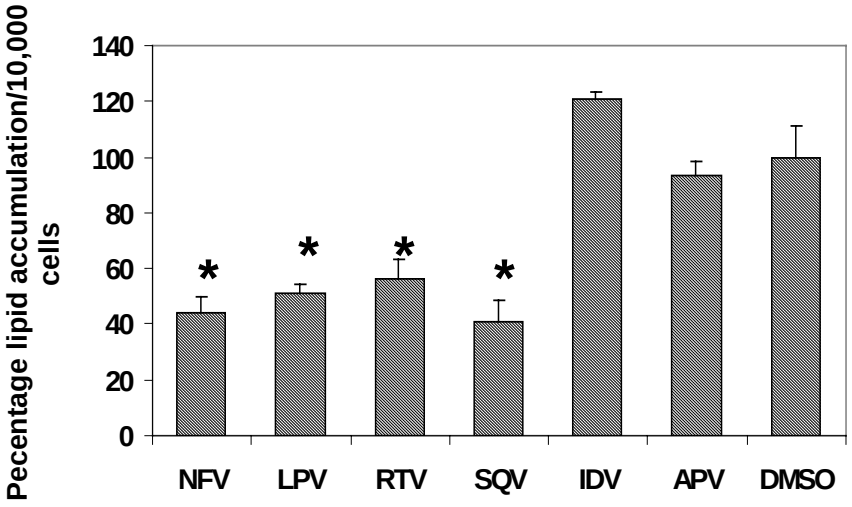
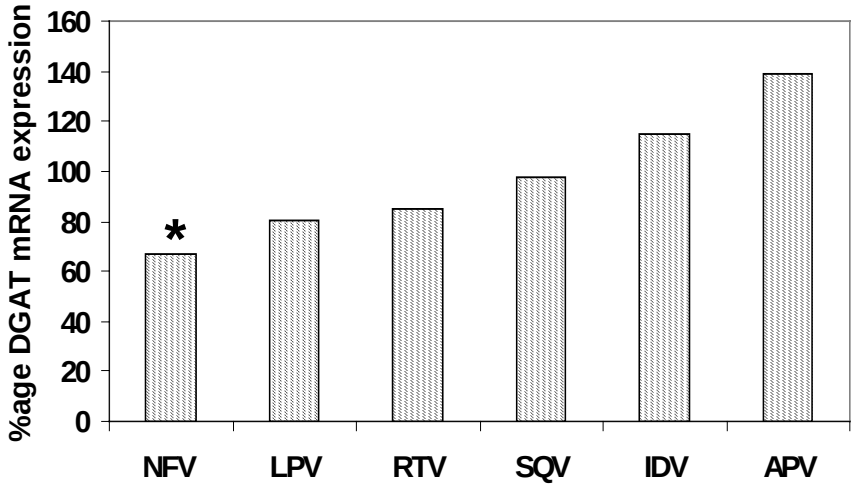
Ritonavir
Nelfinavir
Lopinavir
Amprenavir
Saquinavir

Figure 3A.


Pecentage lipid accumulation/10,000 cells
140
120
100
80
60
40
20
0
*
*
*
*
NFV
LPV
RTV
SQV
IDV
APV
DMSO

B.


%age DGAT mRNA expression
160
140
120
100
80
60
40
20
0
*
NFV
LPV
RTV
SQV
IDV
APV