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Different Impact Of Antiretroviral Drugs On Bone Differentiation In An In Vitro Model

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ABSTRACT

Recently increasing emphasis is placed on preventive health and management of chronic comorbidities avoiding long-term toxicities of antiretroviral therapy (ART).

Drawing from this background we decided to use the Saos-2, osteosarcoma cell line, as a cellular model, to evaluate the effects of some antiretroviral drugs such as abacavir (ABC), tenofovir (TDF), efavirenz (EFV), etravirine (ETR), and darunavir (DRV), on bone differentiation related pathways.

According to our observation, treatment with TDF and ABC affects the ability of the cells to produce calcium deposits with a reduced expression of type I collagen gene and p21 mRNA, also increasing the activity of Wnt3a related pathway. On the other hand treatment with EFV and DRV was not related to any significant reduction of calcium deposits but displayed a decrease in the expression of Wnt3a at day 14 and Type I Collagen at day 7 compared with untreated cells, even if this last down regulation was not confirmed at day 14. Instead ETR administration to Saos-2 cells increases the calcium deposits collagen type I production, as a result of Wnt3a mRNA overexpression, and of an upregulation of collagen type I expression, being also the only drug able to increase the expression of p21 cdk inhibitor as further marker of terminal differentiation.

In summary these data suggest the potential negative interference of TDF and ABC on bone differentiation. DRV and EFV partially affect collagen type I production, instead ETR facilitates a positive bone balance as a result of an increased osteoblasts terminal differentiation. J. Cell. Biochem. 116: 2188–2194, 2015.

KEY WORDS: HIV; HAART; bone; mineralization; osteoporosis

Highly Active Antiretroviral Therapy (HAART) has changed the natural history of Human Immunodeficiency Virus (HIV) infection, delaying the progression to Acquired Immunodeficiency Syndrome (AIDS), significantly increasing the survival of HIV-infected patients [Grigsby et al., 2010b; Focà et al., 2012]. Consequently, increasing emphasis is now placed on preventive

ABBREVIATIONS: HAART, Highly Active Antiretroviral Therapy; HIV, Human Immunodeficiency Virus; AIDS, Acquired Immunodeficiency Syndrome; ART, antiretroviral therapy; BMD, bone mineral density; Fz, frizzled family receptor; ABC, abacavir; TDF, tenofovir; EFV, efavirenz; ETR, etravirine; DRV, darunavir; DMSO, dimethyl sulfoxide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; CKI, cyclin-dependent kinase inhibitor; SD, standard deviation; PI, protease inhibitors; PTH, parathormone; CTX, C-terminal telopeptide of type I collagen; OC, osteocalcin; OPG, osteoprotegerin; sRANKL, soluble receptor activator for nuclear factor kappa β ligand; ALP, alkaline phosphatise.

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health and management of chronic comorbidities avoiding long-term toxicities of antiretroviral therapy (ART), such as renal, and bone diseases [Grigsby et al., 2010b].

The prevalence of osteoporosis is approximately 3-fold higher in HIV-infected patients than in non-HIV-infected patients, and ART-treated subjects are described to reach a 2.5-fold higher prevalence of low bone mineral density (BMD) compared to ART-naïve subjects [Brown and Qapish, 2006]. Furthermore, BMD decreases of 2–6% during the first 2 years of treatment, independently from the selected therapeutic regimen [McComsey et al., 2011] and the clinical expression of osteoporosis is the fragility fracture associated with impaired quality of life and a 20% reduction in survival [Arnsten et al., 2007; Triant et al., 2008].

The bone is a dynamic tissue based on a continuous process of modelling and resorption, which involves osteoblasts and osteoclasts. The osteoblasts, derived from mesenchymal stem cells, regulate the bone building phase through the production and deposition of bone matrix, and its mineralization. The osteoclasts, differently, regulate the bone resorption. Extensive cell signalling between osteoblasts and osteoclasts is required for maintaining a balance in their activities and to ensure the homeostasis of the adult bone [De Crignis et al., 2008; Borderi et al., 2009]. In particular, the Wnt3a signalling pathway is involved in osteogenic differentiation. The Wnt family members are described to play important roles in different aspects of cellular differentiation. They deliver signals from outside the cell through cell surface receptors to the inside of the cell, when they are activated by the binding of a Wnt-protein ligand to a Frizzled family receptor (Fz), able to send the biological signal to the intracellular protein in a dependent or independent β-catenin pathway [Gallant et al., 2004; Kennell and MacDougald, 2005].

The correlation between HIV infection and decrease of osteoblastic activity, with BMD loss and subsequent osteopenia and osteoporosis could be considered as multifactorial [De Crignis et al., 2008; Borderi et al., 2009]. In fact if it has been reported that osteoblast-like cell lines can be infected by HIV, consequently, the bone could be considered an additional potential reservoir of HIV infection, and the HIV-related bone damage may be due to direct loss and functional impairment of osteoblasts [Borderi et al., 2009]. In addition, the use of some antiretroviral drugs seems to contribute to bone metabolism derangement [Brown and Qapish, 2006; De Crignis et al., 2008; Grigsby et al., 2010b; Focà et al., 2012; McComsey et al. 2011].

Drawing from this background we decided to use the Saos-2 osteosarcoma cell line, as a cellular model, to evaluate the effects of some antiretroviral drugs, such as abacavir (ABC), tenofovir (TDF), efavirenz (EFV) etravirine (ETR), and darunavir (DRV), on the bone differentiation related pathways.

MATERIAL AND METHODS

CELLS AND CELL CULTURE

The osteosarcoma cell line Saos-2 was purchased from ATCC (USA) and cultivated, according to the recommendation of the supplier in Dulbecco's Modified Eagle Medium containing 10% FBS, 1% penicillin/streptomycin, 1% L-glutammine. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The media, FBS and antibiotics were purchased from Gibco for passaging; cells were detached with trypsin/EDTA and subsequently replated. For osteogenic differentiation the cells were incubated in differentiation medium (basic medium supplemented with 10 mM-glycerophosphate, 10 mM dexamethasone, and 200 M ascorbic acid) supplemented with the different drugs (EFV, ABC, ETR, DRV, TDF) for 14 days. The medium was then changed every 5 days. During treatment we unplugged a point at 4, 7, 14 days.

CHEMICALS AND TREATMENTS

EFV, TDF, and ABC were purchased from Sequoia Research Product Ltd. ETR was obtained from Janssen Pharmaceutica N.V.-CQC as well as DRV. EFV, DRV, TDF, and ETR were dissolved in Dimethyl sulfoxide (DMSO), while ABC was dissolved in H_2O. All test agents were diluted into culture medium at a final concentration 1 μg/ml throughout the course of differentiation. A preliminary dose response curve was performed to determine the higher concentration of the drugs not toxic for the cells.

ALIZARIN RED S

Calcium deposits, indicators of successful in vitro bone formation, can specifically be stained bright orange-red using Alizarin Red S. After 14 days of treatment cells were stained. Briefly, the cells were fixed in 95% ethanol for 30 min at room temperature. Then cells were washed with PBS and stained with 1% ARS (pH 4.2) for 30 min at room temperature. Quantitative analysis was performed by elution with 10% (w/v) cetylpyridium chloride for 10 min at room temperature, and the OD was measured at 570 nm. Experiments were performed in triplicate

RT-PCR

RT-PCR is the method to determine the transcription of a gene. It may be quantitative since the amplification is proportional to the amount to the template, and then to the initial quantity. Using this assay we evaluated the Frizzled family receptors (Fz), activated by Wnt3, and able to send the biological signal to the intracellular compartment.

Briefly, total RNA was isolated from samples by using a PureLink RNA mini kit (Life Technologies, Europe BV- Monza- Italy) as described by the manufacturer. Genomic DNA was eliminated by RNase-free DNase I treatment (Promega Corporation WI-53711-USA). Reverse transcription was performed using SuperScript VILO master mix. Real Time PCR was carried out using Sybr Select master mix (Applied Biosystem Foster City, CA) on StepOne System (Applied Biosystem). The expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization of gene expression values. Melting curve analysis was always performed at the end of each PCR assay to control primer specificity. Relative fold change was calculated by $2^{-\Delta\Delta Ct}$ method. Primers used for qRT-PCR are as follows: hFz1 forward 5′-GTGAGCCGACCAAGGTGTAT-3′, hFz1 reverse 5′-CAGCCGGACAAGAAGATGAT-3′; hFz2 forward 5′-GCGTCTTCTCCGTGCTCTAC-3′, hFz2 reverse 5′-CTGTTGGTGAGGCGAGTGTA-3′; p21 forward 5′-GCAGACCAGCATGACAGATTT-3′;

p21reverse 5′-GGATTAGGGCTTCCTCTTGGA-3′ obtained from Eurofins mwg/operon.

Real-time PCR was conducted in a volume of 25 μl containing 40 ng cDNA (1/100 dilution of reverse transcriptase mixture), 1.25 μl of different primers (Fz1, Fz2, p21) and 12.5 μl TaqMan Universal PCR Master Mix (Applied Biosystems) in the following sequence: 2 min at 50°C and denaturation for 10 min at 95°C followed by 40 cycles of the amplification step at 95°C for 15 s (denaturation), and then at 60°C for 60 s (annealing/extension) in 96-well plates with the ABI PRISM 7000 sequence Detection System (Applied Biosystems). Real-time PCR for the endogenous control GADPH was carried out under the same conditions, using a GADPH Assay on Demand (Applied Biosystems). A standard curve for the different genes was constructed using serial dilutions (200–40–8–1.6 ng) of a pool of cDNA produced from RNA extracted from the cells treated with different groups of drugs (ABC, DRV, TDF, EFV, ETR). Results have been analyzed using the Applied Biosystems analysis software and expression levels were calculated from a linear regression of the standard curve. Results are given as Fz1, Fz2, p21 expression vs. GADPH expression to correct for differences in the quantity of cDNA used in the PCR reaction. All real-time PCR reactions for each sample were performed in triplicate.

SEMIQUANTITATIVE (SQ) RT-PCR

The sqRT-PCR is a technique designed to quantify gene expression. This technique is able to give reasonable estimates of the expression levels of the messenger amount. The first step is to perform few cycles of amplification. The amplification products are resolved on agarose gel and the result is displayed using a radioactive probe by Southern blotting.

Briefly, for gene-specific RT-PCRs, each reaction was prepared with 5 μl cDNA, 2.5 μl 10× PCR buffer (Mg^{2+}-free), 0.75 μl 50 mM $MgCl_2$, 0.5 μl 10 mM dNTPs, 0.5 μl 10 mM primer mix, 0.1 μl Taq polymerase (5 U/μl; all solutions from Invitrogen Life Technologies, Europe BV-) in a total volume of 25 μl. PCR cycles were 2 min denaturing step at 94°C, followed by 40 cycles of 94°C for 30 s, 60°C for 60 s, 72°C for 30 s, and a final extension step at 72°C for 4 min. Primer sequences were hWnt3a forward 5′-TGTTGGGCCACAG-TATTCCT-3′, hWnt3a reverse 5′-ATGAGCGTGTCACTGCAAAG-3′. The RT-PCR conditions and cycle numbers were chosen so that none of the gene-specific amplicons reached a plateau at the end of the PCR protocol, i.e., they were in the exponential phase of amplification. Functionality of primers was tested on cDNAs obtained from different tissues prior to the experiments to exclude false-negative results. 10 μl of the PCR were loaded on a 1.5% agarose gel and after electrophoresis, PCR products were visualized by ethidium bromide staining. Fluorescence signals were detected with a Lumi imager (Boehringer, Milan - Italy) and quantification was performed with the Lumi-Analyst software (Boehringer). Band intensities were expressed as relative absorbance units. The ratio between the gene-specific PCR amplification product and reference gene GAPDH was calculated to normalize for initial variations in sample concentration and as a control for reaction efficiency. Mean and standard deviation (SD) of all experiments were calculated after normalization to GAPDH. Experiments were performed in triplicate.

IMMUNOBLOTTING

Immunoblotting techniques use antibodies to identify target proteins among a number of unrelated protein species via antigen-antibody specific reactions. Proteins are lysed and separated by electrophoresis and transferred onto membranes. Using this assay we evaluated the Type I Collagen, a late marker of osteogenic differentiation.

Briefly, plates of 70–80% confluent Saos-2 cells were treated for 4–7–14 days. Then the cells were collected. Briefly, the cells were lysed in 100 μl lysis buffer (RIPA lysis buffer system Santa Cruz Biotechnology, Inc, CA) for 30 min in ice. Lysates were centrifuged at 14,000*g* for 10 min at 4°C. Total cell protein extracts were normalized for concentration by the Bradford assay (Bio-Rad Laboratories Segrate Milan Italy) and 20 μg of proteins were separated by SDSPAGE and transferred to polyvinylidene difluoride membrane (Millipore′s Corporate, Billerica, MA). Membranes were incubated with rabbit polyclonal anti-collagen type I (EMD Millipore USA Calbiochem, cat. 234167). Primary antibodies were detected using anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Inc., Piscataway, NJ) and visualized by the ECL detection system (Amersham Biosciences, Inc.) according to the manufacturer′s instructions. Each membrane was probed with the monoclonal anti-actin antibody (Fitzgerald Industries International USA, cat.10R-2380) to estimate equal protein loading.

Quantitation of membranes was made using ImageJ software.

STATISTICAL ANALYSES

Where applicable, numerical results were expressed as the mean ± SEM of three independent experiments. Statistical differences were considered if the P value was <0.05 as determined by ANOVA followed by Student′s t-test. Data are expressed as the percentage and SEM.

RESULTS

In order to evaluate the influence of five anti-HIV drugs (ABC, TDF, EFV, ETR, DRV) on bone differentiation, a preliminary dose-response curve was performed to determine the concentration of the drugs that was not toxic for the cells, using Saos-2, osteosarcoma cell line as a cellular model.

The cells were subjected to treatments with drug concentrations of 0.5, 1.0, and 1.5 μg/ml for 48 h. Samples were collected at 8, 12, 24, 36, 48 h, and a FACS analysis was performed in order to assess the vitality and the threshold limit of toxicity of drugs. The data obtained established that the concentration of 1 μg/ml was the best as non-toxic and capable of exerting an effect on the cell cycle in a reasonable time of 48 h. Moreover, from the same analysis, we observed that some of these drugs were able to reduce cell growth in the cell line, with a stable effect during prolonged exposure (data not shown).

In order to evaluate the effects of these drugs on osteogenic differentiation, we used Saos-2 cells treated with differentiation medium supplemented with the above mentioned different drugs at the concentration of 1 μg/ml for 14 days. Medium was changed every 5 days. Figure 1a shows plates at 14 day after differentiation

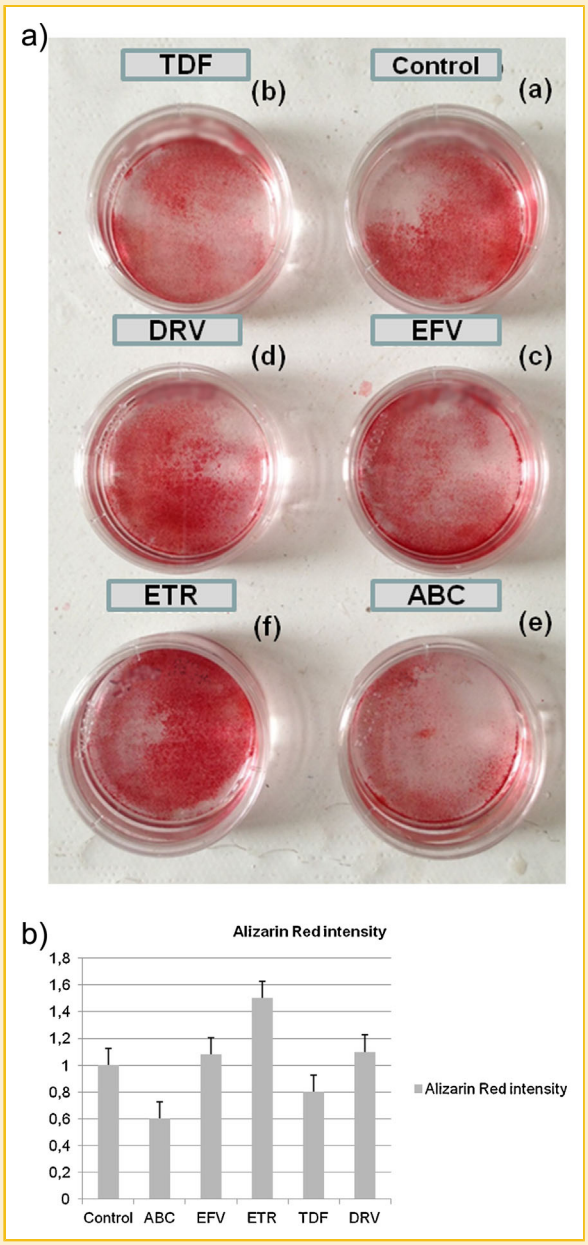

Fig. 1. (a) Formation of calcification nodules in each group in Saos-2 cells. Saos-2 cells were cultured with or without treated materials for 14 days and stained with Alizarin red. A representative photograph of Alizarin red staining is shown, (a) control; (b) TDF; (c) EFV; (d) DRV; (e) ABA; (f) ETR; (b) Alizarin red analysis of calcification staining.

stained with Alizarin-Red S. Observing calcium deposits, as an index of successful in vitro bone formation, we found that TDF and ABC were able to inhibit the calcium deposit, whereas EFV and DRV did not show any significant increase of calcium deposits while ETR increased the calcium deposits compared with untreated cells. Figure 1b shows the quantitation of alizarin red analysis of calcification staining.

In order to confirm the morphological data obtained with the Alizarin-Red S stain, we performed an immunoblotting with Type I Collagen, a late marker of osteogenic differentiation, on cells treated with differentiation medium supplemented with the drugs (Fig. 2a); instead figure 2b shows a diagram that quantify the intensity of the immunoblotting. The results showed a decrease in the expression of Type I Collagen in the cells treated with TDF and ABC compared with untreated cells. In particular TDF was able to slowly decrease in the expression of Type I Collagen, alternatively ABC at the beginning of the treatment increased the expression of Type I Collagen, instead, at the end of the treatment, it was able to decrease the expression of Type I Collagen.

The cells treated during the differentiation with EFV and DRV showed a decrease in the expression of Type I Collagen at day 7 compared with untreated cells. Instead, at day 14 the expression levels of Type I Collagen in the cells treated and untreated were similar, thus supporting the ipothesis that the differentation is slowed.

On the other hand we observed an increase of expression of collagen type I in the cells treated with ETR compared with the untreated cells. This increase does not appear at the beginning of the treatment but at the end of the differentiation. Interestingly at the beginning of the treatment the expression seems to be inhibited, reaching at day 7 the same level of the control and an increase at the end of the differentiation.

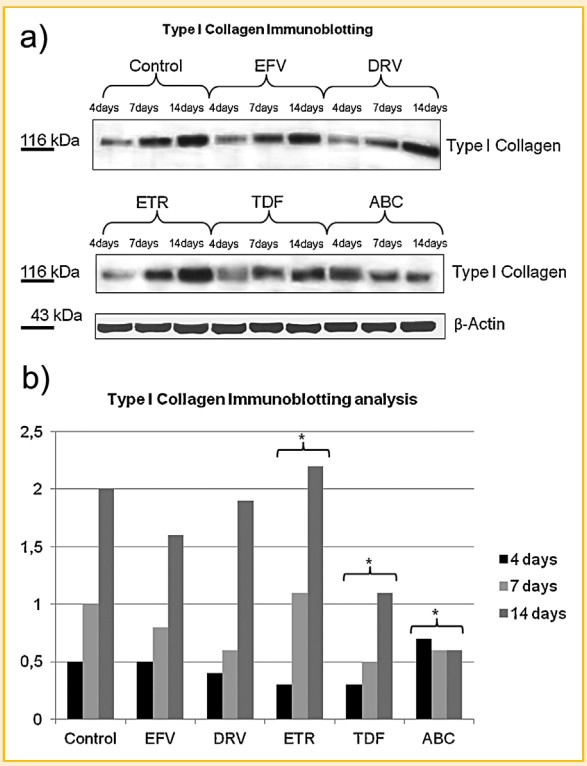

Fig. 2. (a) Immunoblotting of Saos-2 lysates cells (20 μg proteins) treated for 4-7-14 days with the drugs, using anti-Type I Collagen antibody; (b) Immunoblotting analysis of Type I Collagen protein expression levels. Data are expressed as mean ± standard deviation of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ ANOVA.

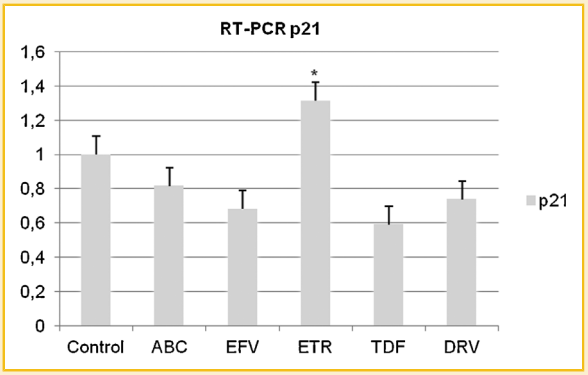

Fig. 3. Total RNA was prepared from the Saos-2 cells treated for 14 days with the drugs, and was analyzed by RT-PCR. The level of each mRNA is shown relative to the level of p21 mRNA. Data are expressed as mean ± standard deviation of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ ANOVA.

Consistent with the data obtained by Alizarin-Red S stain and immunoblotting, only ETR was able to increase significantly the mRNA expression of p21 a cyclin-dependent kinase inhibitor (CKI) able to regulate cell cycle progression and involved in the process of differentiation (Fig. 3), while, looking at the cells treated with the other four drugs, we observed a decrease of p21 expression.

Furthermore we generated primers to estimate, by semiquantitative RT-PCR at day 14, the expression of Wnt3a on cells treated with differentiation medium supplemented with the different drugs. Figure 4a points out that ABC, ETR, and TDF were able to upregulate Wnt3a protein expression, instead EFV and DRV were able to downregulate Wnt3a expression. Figure 4b shows a diagram that graphically quantify semiquantitative RT-PCR data.

Moreover we evaluated also the two Frizzled family receptors able to bind Wnt3a by RT-PCR at 14 day. In detail, EFV, TDF e DRV were specifically able to increase the expression only of Fz2 compared with the untreated cells even with quantitative differences (Fig. 5). Also cells treated with ETR displayed a slight Fz2 up regulation, while cells treated by ABC showed a slight downregulation of this last protein expression. The levels of expression of Fz1 were not affected by treatment with any of the evaluated drugs compared with control.

DISCUSSION

In this report we investigated in vitro effects of anti-HIV drugs, such as ABC, TDF, EFV, ETR, and DRV, on bone differentiation. We chose Saos-2 cells as an in vitro model to assess the effects of these different drugs on bone homeostasis.

Recent clinical comparative studies reported that the greater decrease of BMD and increase in bone turnover are associated to TDF [Gallant et al., 2004; Stellbrink et al., 2010; McComsey et al. 2011; Haskelberg et al., 2012] and protease inhibitors (PI)-containing ART regimen [Focà et al., 2012]. Consistently, the use of specific antiviral drugs, such as TDF and PIs, correlates to the changes in plasma concentrations of bone turnover biomarkers, such as C-terminal

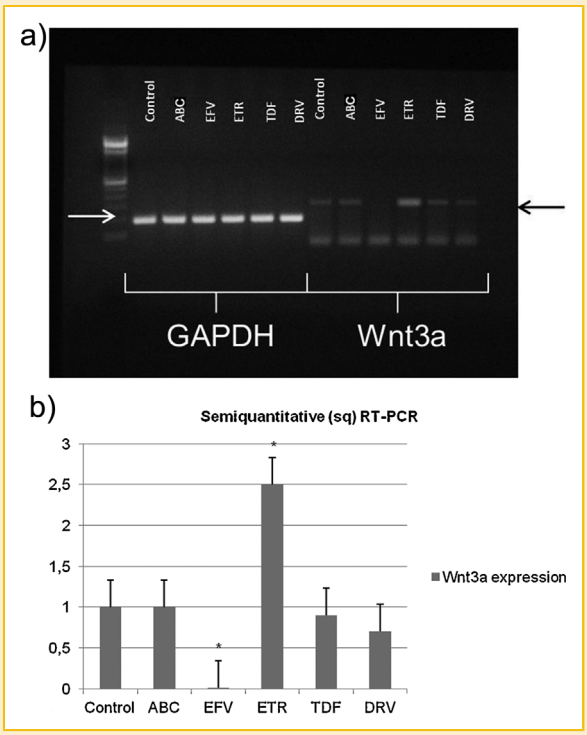

Fig. 4. (a) Semi-quantitative RT-PCR of Wnt3a in Saos-2 cells treated for 14 days with the drugs. The PCR was terminated after 40 cycles and was normalized with GAPDH; (b) Semi-quantitative RT-PCR quantification analysis. Data are expressed as mean ± standard deviation of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ ANOVA.

telopeptide of type I collagen (CTX), osteocalcin (OC), osteoprotegerin (OPG), the receptor activator for nuclear factor kappa β ligand (RANKL), and inflammatory cytokines [Gibellini et al., 2010; Brown et al., 2011; Cotter et al., 2013]. In more detail DRV has been related to the specific increase of RANKL expression and to the induction of

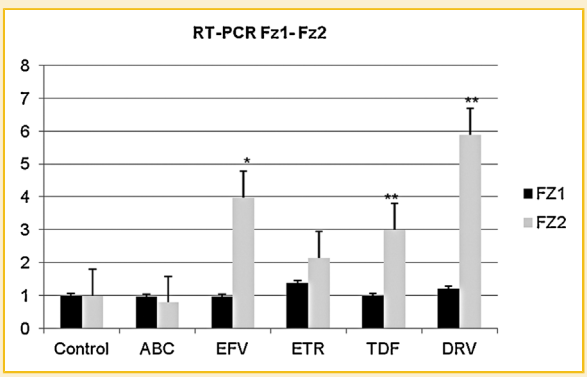

Fig. 5. Total RNA was prepared from the Saos-2 cells treated for 14 days with the drugs, and was analyzed by RT-PCR. The level of each mRNA is shown relative to the level of Fz1 and Fz2 mRNA. Data are expressed as mean ± standard deviation of triplicate experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ ANOVA.

osteoclastic activity with bone resorption in chronic treatment [Gibellini et al., 2010]. The decrease of BMD as well as the increase of bone turnover markers due to TDF-based therapy are not completely explained by a compromised renal function [Rasmussen et al., 2012], while ABC administration correlates to a low BMD and to a significant spine bone density reduction [Kim et al., 2013]. EFV, instead, has not yet been directly associated to a decrease of BMD, but to an increase of vitamin D catabolism, and therefore to a severe vitamin D deficiency [Welza et al., 2010; Etminani-Esfahani et al., 2012].

ETR appears to have a positive activity on bone turnover, but there are not similar observations in vivo. Clinical data should be obtained to confirm the activity of ETR on the bone balance, in order to consider these data useful to choose the best tailored therapeutic approach.

Even if in the overall literature there are many clinical observations about the relationships between the bone metabolism and some antiretroviral drugs, the involved pathogenic mechanisms have not yet completely been elucidated. Few studies analyzed the possible ability of some antiretroviral drugs to interfere with cell cycle related pathways and most in vitro experiments on osteoblasts and osteoclasts were only focused on TDF activities [Grigsby et al., 2010a, b]. In more detail an in vitro study on primary murine osteoblasts demonstrated that TDF downregulated the expression of genes involved in six different signalling pathways: Wnt (frizzled homolog 4, frizzled related protein, Rac3), transforming growth factor-beta (TGF-β), Hedgehog (hedgehog-interacting protein), MAPK (Rac3), VEGF (Rac3), B cell receptor (Rac3), and the Fc epsilon RI signalling pathway (Rac3). These pathways are involved in amino acid biosynthesis and metabolism and expecially secreted frizzled-related proteins have been reported to inhibit osteoblasts differentiation and their observed downregulation by TDF could result in reduction of osteoblasts growth, activities, and differentiation and in decrease of bone formation with reduced BMD [Grigsby et al., 2010a]. Furthermore, TDF exposure on primary murine osteoclasts downregulates the expression of Gnas, Got2 (mitochondrial enzyme involved in energy transduction), Ass1, and Pycr1, involved in amino acid metabolism, and Snord32a, resulting in less MAPK/ERK with consequent increase of bone resorption [Grigsby et al., 2010a].

The effects of different PI on bone metabolism have been also studied in vitro on primary human osteoblast. They are reported to induce significant changes in genotypic expression, such as TIMP-3, which is involved in osteoblast differentiation and extracellular matrix development processes, and decrease Runx-2 mRNA expression, calcium deposition and alkaline phosphatase (ALP) activity [Malizia et al., 2007].

In another study on ex vivo cultured osteoclasts, osteoblasts, and adipocytes, it has been demonstrated that PI interferes with the osteoclasts activity and decrease osteoblast ALP activity, significantly reducing calcium deposition, and osteoprotegrin expression [Jain et al., 2002].

However these in vitro data are still insufficient to clarify the complex interactions between the antiretroviral drugs and the bone metabolism. As above reported we have quite a good knowledge of TDF and its activity, while few informations about ABC, EFV, ETR, and DRV are currently available.

TDF and ABC had very similar effects on bone metabolism. These data correlate with previous clinical observations. Even if mainly PI and TDF have been strongly associated with bone loss, also ABC induces bone turnover, probably for its pro-inflammatory properties [Kim et al., 2013]. In addition an in vivo different effect of TDF and ABC, could be explained by the interactions with other ARV drugs, not analyzed in vitro, as described in literature [Stellbrink et al., 2010], and by the renal impairment more frequently observed in patients treated by TDF.

The aim of our study was to contribute to the knowledge of the effects of these drugs on the production of calcium deposits, the expression of type I Collagen, p21 mRNA expression and the activity of Wnt3a pathway.Considering that the pathway involved in bone differentiation are more than one we decided to be focused in this first step of our project to evaluate the effects of Wnt and in particular of Wnt3a since this is a protein family strictly involved in the differentiation processes [Ambrosetti et al., 2008; Okoye et al., 2008; Zhang et al., 2013].

According to our observation and consistently to previous clinical reports [Brown and Qapish, 2006; Grigsby et al., 2010; McComsey et al., 2011; Focà et al., 2012], treatment with TDF, and ABC affects the ability of the cells to produce calcium deposits with a reduced expression of type I collagen gene and p21 mRNA.

On the other hand treatment with EFV and DRV is not related to any significant reduction of calcium deposits, but displays a decrease in the expression of Wnt3a at day 14 and Type I Collagen at day 7 compared with untreated cells, even if this last downregulation was not confirmed at day 14. Furthermore, ETR administration to Saos-2 cells increases the calcium deposits collagen type I production, as a result of an upregulation of collagen type I expression, with Wnt3a mRNA overexpression, being also the only drug able to increase the expression of p21 cdk inhibitor as further marker of terminal differentiation. Since p21 is involved in numerous cellular processes, in particular the cell cycle and differentiation, it is clear the possibility of being able to assume an action of ETR in promoting osteoblastic differentiation. In fact it is seen that the expression of p21 can be increased in differentiation processes; in particular, in muscle differentiation, this increase mediated by MyoD [Guo et al., 1995].

EFV, TDF and DRV and slightly ETR but not ABC were specifically able to increase the expression only of Fz2 that seems to be the selective Wnt3a ligand in our subset.

These data suggest the potential negative interference of TDF and ABC on bone differentiation resulting in an upregulation of Wnt3a as a Fz2 mediated ineffective answer to the bone balance derangement. The administration of DRV and EFV seems also to partially affect collagen type I production, at least in an initial phase of our experiments, while ETR seems to facilitate a positive bone balance as a results of an increased osteoblasts terminal differentiation with all the evaluated pathways contributing to this effect. However future studies might be focused on understanding which mechanisms are involved and establishing the pathways involved in bone differentiation.

Obviously these results should be related to the clinical observations. More powerful studies are, then, needed to correlate

changes in bone turnover markers and gene expression with bone metabolism and low BMD, to clarify the clinical effects of ART, and possible markers of bone turnover.

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