Analysis of the effects of specific protease inhibitors on OPG/RANKL regulation in an osteoblast-like cell line

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SUMMARY

Bone mass loss with the subsequent development of osteopenia and osteoporosis is related to HIV infection and antiretroviral treatment, even though the mechanisms involved have not yet been elucidated.
In this report analyzes the early effects of some specific protease inhibitors on OPG/RANKL yielding and cell survival in osteoblast-like HOBIT cell line. None of the compounds, tested at scalar concentrations (C1, C2, C3), affected cell survival except for tipranavir that elicited a reliable induction of apoptosis at the highest concentration (C3). Atazanavir, saquinavir and indinavir did not affect OPG/RANKL in the cell surnatant in our experimental conditions. By contrast, at optimal concentration (C2), fosamprenavir induced a significant increase in OPG associated with a RANKL decrease whereas tipranavir down-regulated both OPG and RANKL (at C2 and C3) and darunavir increased RANKL only at C3 concentration.
Together these data (coupled with the analysis of OPG/RANKL ratio) indicate that at early times and at optimal concentrations the PIs did not impair the OPG/RANKL system with the exception of fosamprenavir that showed a relative positive OPG/RANKL ratio regulation. Instead, cell cultures treated by the highest concentrations of tipranavir or darunavir showed a change in cell survival or an increase in RANKL, with a negative effect on the OPG/RANKL balance.

KEY WORDS: HIV, Protease inhibitors, OPG/RANKL

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INTRODUCTION

The advent of highly active antiretroviral therapy (HAART) in treatment of HIV infection has revolutionized the pharmacological and clinical management of HIV positive patients (Fauci, 2003).
HAART inhibits viral replication causing a plasma viral load decrease that can be observed for a long time. Although HAART is a key element to tackle the progression of HIV-related disease, this therapeutic approach has some drawbacks. Firstly, HAART does not eradicate HIV infection because HIV persists actively in the viral reservoirs represented by memory CD4+ T lymphocytes and monocytes/macrophages (Re *et al.*, 2006). Secondly, HIV-infected patients treated by HAART can exhibit resistance to specific antiretroviral compounds arising during treatment. Thirdly, HAART may determine a pharmacological-related toxicity in several tissues and organs (Fauci 2003, Haggerty *et al.,* 2006, Levy 2009, De Crignis *et al.*, 2008). In spite of these problems, HAART has radically modified the clinical focus of HIV infection through its consistent effects on

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the patient. Whereas HIV infection was a progressively lethal disease before the HAART era, it can now be considered as a chronic pathology where HAART can slow its development. Physicians once focused their attention almost exclusively on the basic topics of the immunity system, viral load monitoring and the surveillance and treatment of opportunistic diseases. Today they have to take into account HIV-related damage to organs and tissues and the effects of HAART toxicity.

In particular, lesions to the CNS, heart, kidney and bone are increasingly monitored by physicians. Bone derangement was already known in the pre-HAART era: some studies indicated a direct correlation between HIV infection and bone loss with subsequent osteopenia and osteoporosis (Serrano *et al.,* 1995, Mondy and Tebas 2003, Borderi *et al.,* 2009). The classical review by Brown and Qaqish (Brown and Qaqish, 2006) is a metanalysis of the most significant reports published from 1994 to 2005 on HIV/bone interaction. The report clearly demonstrated that naive HIV-infected patients have increased odds of osteopenia (6.4 fold) and osteoporosis (3.2 fold) than HIV uninfected subjects.

The mechanism(s) involved in bone impairment is (are) not well known: the homeostasis of bone mass in adult individuals is the result of a well-tuned balance of osteoblast-related bone synthesis and osteoclast-related bone resorption also regulated by several hormones, vitamins and cytokines (Borderi *et al.,* 2009). In particular, the biological pathway represented by the RANKL/RANK/OPG system plays an essential role in bone metabolism control. RANKL is a protein produced by osteoblasts and T lymphocytes stimulating the differentiation and activity of osteoclasts by binding with RANK, a receptor detectable on the osteoclast cell membrane. RANKL activity is modulated negatively by a soluble molecule OPG, which inhibits RANKL/RANK binding (Marie 2008). Although HIV cannot directly infect osteoblasts, HIV can impair bone homeostasis (Nacher *et al.,* 2001, Gibellini *et al.,* 2008) by gp120/cell membrane interaction inducing apoptosis by paracrine/autocrine TNF-α activation. In addition, several studies have shown that HIV-positive naive subjects display a plasma RANKL increase with a decrease of the OPG/RANKL plasma ratio. These changes in the RANKL and OPG/RANKL plasma ratio could suggest an impaired functional balance between osteoblasts and osteoclasts that may explain the possible bone derangement (Koishi *et al.,* 2005, Gibellini *et al.,* 2007, Mora *et al.,* 2007).

The role of HAART in the genesis or enhancement of osteopenia and osteoporosis has been investigated by several groups, but controversial data were obtained because different experimental conditions, pharmacological associations and statistical approaches were applied to these studies. A statistical meta-analysis (Brown and Qaqish 2006) demonstrated that HIV positive HAART treated patients have increased odds (2.4 fold) of osteoporosis with respect to HIV positive naive individuals, and PI treatment increases the osteoporosis odds 1.6 fold in comparison with patients treated without PIs.

This study tested several protease inhibitors such as fosamprenavir, saquinavir, indinavir, darunavir, tipranavir and atazanavir on a cellular model represented by an osteoblast-like HOBIT cell line to shed light on the effects of these molecules on bone homeostasis through the analysis of RANKL and OPG in the cell culture surnatants.

MATERIALS AND METHODS

The osteoblast-like human HOBIT cell line was kept in DMEM/F12 medium (Gibco, Paisley, UK) plus 10% of fetal calf serum (FCS; Gibco), antibiotics and aminoacids (Baldini *et al.* 2008, Gibellini *et al.,* 2008). Cell cultures at approximately 70% of cell confluence were treated by scalar concentrations of fosamprenavir (Glaxo Smith & Kline UK; 1.4, 7 and 35 μg/ml), tipranavir (Boheringer Ingelheim, Ingelheim, Germany; 1, 5 and 25 μg/ml), Darunavir (Janssen-Cilag Ltd, Beerse, The Netherlands; 1.8, 9 and 45 μg/μl), saquinavir (Roche; 0.6, 3 and 15 μg/ml), Indinavir (Merck-Sharp and Dohme; 1, 5 and 25 μg/ml) and atazanavir (Bristol-Myers Squibb, Uxbridge UK; 0.6, 3 and 15 μg/ml). The commercial pharmacological compounds were dissolved in DMSO. The mean concentration (C2) of the tested molecules is the concentration detectable in the plasma of in vivo treated subjects (Electronic Medicines Compendium). The surnatants of cell cultures were analyzed at 24 and 48 hours after the start of treatment for

RANKL and OPG using two commercial kits represented by RANKL detection kit (Peprotech, London, UK) and Duo ELISA OPG kit (R&D, Minneapolis, MN). The analysis was carried out following the manufacturers' instructions. The negative control was represented by untreated HOBIT cell cultures kept in the cell medium plus opportune DMSO amount. The apoptosis analysis by iodium propide staining was described in (Gibellini *et al.,* 2008). In brief, the cells were detached by trypsin (Gibco), washed in PBS and fixed in cold 70% ethanol for 60 min at 4°C. After the fixation step, the cells were washed in PBS and treated with 0.5 mg/ml of RNase A (Roche, Mannheim, Germany) for 1 h at 37°C. Propidium iodide (PI; 40 mg/ml in PBS; Sigma) was then added to each sample in the dark at 4°C for 10 min and then measured in flow cytometry using FACScan flow cytometer (Becton-Dickinson, Palo Alto, CA.) equipped with an Argon laser tuned at 488 nm. The analysis was performed by Lysis II software (Becton Dickinson). All data were expressed as mean ± standard deviation (SD) of three separate experiments performed in duplicate. Statistical analysis was carried out with the Wilcoxon test.

RESULTS

Three specific scalar concentrations (C1, C2, and C3) of atazanavir, darunavir, indinavir, tipranavir, fosamprenavir and saquinavir were selected: C2 is the mean concentration and is considered the optimal concentration because it is the specific plasma concentration of each compound generally detected in the HIV positive treated patients (Electronic Medicines Compendium). C1 represents the lowest concentration (fivefold less than C2) whereas C3 is the highest (fivefold more than C2).

In the first group of experiments, the selected protease inhibitors were analyzed to determine whether they can induce apoptosis in the HOBIT cell cultures. The cell cultures were analyzed by flow cytometry iodium propide staining at 24-48 hours after the start of the treatment. Atazanavir, indinavir, tipranavir, fosamprenavir, darunavir and saquinavir did not show significant apoptosis at any of the three concentrations tested (Table 1) whereas tipranavir, at C3 concentration showed an increase in apoptosis induction both at 24 and 48 hours (7%±1.5 vs 2.9%±1.2 at 24 hours e 10.9%±4 vs 4,1%±2.3 at 48 hours).

In the second group of experiments, RANKL and OPG were analyzed in the cell surnatants at early times (24-48 hours) after the start of treatment. OPG content was never significantly affected by indinavir, atazanavir, darunavir or saquinavir at all tested concentrations and times. On the other hand, fosamprenavir increased OPG in the HOBIT cell culture surnatants at 24-48 hours when C2 and C3 concentrations were employed. Fosamprenavir C2 and C3 treatments induced a 90% increase in OPG (C2: 3530±847 vs 1858±241; $p<0.05$) and a 47% increase (2731±519 vs 1858±241; $p<0.05$) at 24 h, respectively, when compared with the cell culture controls. Likewise, fosamprenavir confirmed the OPG induction at 48 hours with C2 and C3 concentrations (C2: 2905±523 vs 1760±334; $p<0.05$; C3: 2728±400 vs 1760±334; $p<0.05$). Tipranavir was the only com-

TABLE 1 - *Apoptosis detection in HOBIT cell cultures at different experimental times.*

	24 hours	*48 hours*
Untreated	2.9±1.2	4.1±2.3
Indinavir 1 µg/ml	2.4±1.3	3.8±2.0
Indinavir 5 µg/ml	2.8±1.5	3.7±3.1
Indinavir 25 µg/ml	3.9±1.7	4.7±3.4
Saquinavir 0.6 µg/ml	2.3±1.0	4.2±2.5
Saquinavir 3 µg/ml	3.0±0.9	4.8±2.0
Saquinavir 15 µg/ml	3.6±1.8	5.3±2.7
Fosamprenavir 1.4 µg/ml	2.7±1.5	4.0±1.5
Fosamprenavir 7 µg/ml	3.3±1.5	4.5±3.4
Fosamprenavir 35 µg/ml	3.5±1.8	5.1±2.3
Atazanavir 0,6 µg/ml	3.0±1.3	4.4±1.9
Atazanavir 3 µg/ml	3.2±1.2	4.9±2.8
Atazanavir 15 µg/ml	4.1±1.1	5.3±2.4
Tipranavir 1 µg/ml	3.5±1.6	5.1±3.1
Tipranavir 5 µg/ml	5.0±3.4	6.9±3.4
Tipranavir 25 µg/ml	7.0±1.5*	10.9±4.0*
Darunavir 1.8 µg/ml	3.1±2.1	4.4±2.0
Darunavir 9 µg/ml	3.8±2.3	5.7±2.9
Darunavir 45 µg/ml	4.7±2.9	7.1±3.2

The data (%) are expressed as mean ± SD of three experiments performed in duplicate. *($p<0.05$; Wilcoxon test).

pound that induced a decrease of OPG using C2 and C3 concentrations both at 24 and 48 hours ($p<0.05$; Table 2).

The ELISA analysis of RANKL (Table 3) in the cell surnatants showed that only fosamprenavir, tipranavir and darunavir affected RANKL amount whereas saquinavir, atazanavir and indinavir did not exhibit any significant effect in any of the tested experimental conditions. In particular, fosamprenavir significantly decreased RANKL only using C2 concentration both at 24 hours 162+24 vs 267±59; $p<0.05$) and at 48 hours 164+21 vs 274±44; $p<0.05$) whereas at C3 the decrease of RANKL was detectable but not significant. Tipranavir decreased RANKL using C2 and C3 concentrations both at 24 hours (C2: 141±11 vs 267±59; $p<0.05$; C3: 88±10 vs 267±59; $p<0.05$) and 48 hours (C2: 151±15 vs 274±44 $p<0.05$; C3; 85±11 vs 274±44; $p<0.05$). In contrast with fosamprenavir and tipranavir, darunavir increased RANKL surnatant content, but it was a transitory effect because it was detectable only using C3 at 24 hours (412±83 vs 267±59 $p<0.05$).

The OPG/RANKL ratio may be considered a useful parameter to monitor bone homeostasis. A low plasma OPG/RANKL ratio has been related to lower bone mass in some cases of HIV positive naive patients (Gibellini *et al.,* 2007).

The OPG/RANKL ratio, calculated from the mean data of OPG and RANKL, indicated (Table 4) that only fosaprenavir determined a sharp increase in this ratio. Fosamprenavir C2 showed a ratio of 21.8 versus the 6.9 ratio of the control at 24 hours and a ratio of 17.7 versus 6.4 at 48 hours due to the concomitant increase in OPG and decrease of RANKL. In addition, tipranavir showed a moderate increase in ratio although both RANKL and OPG dropped at lower levels whereas atazanavir and darunavir showed a small reduction of ratio at specific experimental conditions (atazanavir C3 at hours 4.3 vs 6.9 and darunavir C3 at 24 hours 4.9 vs 6.9).

TABLE 2 - *OPG content in HOBIT cell culture supernatants at different experimental times.*

	24 hours	*48 hours*
Untreated	1858±241	1760±334
Indinavir 1 µg/ml	1597±351	1654±297
Indinavir 5 µg/ml	2080±291	1900±285
Indinavir 25 µg/ml	2192±482	1954±254
Saquinavir 0.6 µg/ml	1725±328	1742±261
Saquinavir 3 µg/ml	1672±385	1812±362
Saquinavir 15 µg/ml	1861±298	1830±201
Fosamprenavir 1.4 µg/ml	2197±241	2094±188
Fosamprenavir 7 µg/ml	3530±847*	2905±523*
Fosamprenavir 35 µg/ml	2731±519*	2728±400*
Atazanavir 0.6 µg/ml	2044±225	1795±269
Atazanavir 3 µg/ml	1783±392	1813±344
Atazanavir 15 µg/ml	1579±158	1655±364
Tipranavir 1 µg/ml	1412±367	1478±325
Tipranavir 5 µg/ml	1337±174*	1373±288*
Tipranavir 25 µg/ml	873±148*	757±75*
Darunavir 1.8 µg/ml	2063±433	1725±207
Darunavir 9 µg/ml	2007±261	1690±186
Darunavir 45 µg/ml	2025±182	1830±348

The data (ng/ml) are expressed as mean ± SD of three experiments performed in duplicate. *($p<0.05$; Wilcoxon test).

TABLE 3 - *RANKL content in HOBIT cell culture supernatants at different experimental times.*

	24 hours	*48 hours*
Untreated	267±59	274±44
Indinavir 1 µg/ml	310±46	299±30
Indinavir 5 µg/ml	339±58	320±61
Indinavir 25 µg/ml	350±84	331±89
Saquinavir 0.6 µg/ml	270±24	282±37
Saquinavir 3 µg/ml	262±39	279±22
Saquinavir 15 µg/ml	323±40	285±46
Fosamprenavir 1.4 µg/ml	238±55	255±51
Fosamprenavir 7 µg/ml	162±24*	164±21*
Fosamprenavir 35 µg/ml	190±23	207±64
Atazanavir 0.6 µg/ml	291±29	279±48
Atazanavir 3 µg/ml	299±69	293±82
Atazanavir 15 µg/ml	363±98	323±93
Tipranavir 1 µg/ml	198±49	219±46
Tipranavir 5 µg/ml	141±11*	151±15*
Tipranavir 25 µg/ml	88±10*	85±11*
Darunavir 1.8 µg/ml	254±58	288±47
Darunavir 9 µg/ml	232±69	282±62
Darunavir 45 µg/ml	412±83*	331±79

The data (ng/ml) are expressed as mean ± SD of three experiments performed in duplicate. *($p<0.05$; Wilcoxon test).

TABLE 4 - *OPG/RANKL mean ratio evaluation at different experimental times.*

	24 hours	*48 hours*
Untreated	6.9	6.4
Indinavir 1 µg/ml	5.2	5.5
Indinavir 5 µg/ml	6.2	5.9
Indinavir 25 µg/ml	6.3	5.9
Saquinavir 0.6 µg/ml	6.4	6.2
Saquinavir 3 µg/ml	6.5	6.5
Saquinavir 15 µg/ml	6.0	6.4
Fosamprenavir 1.4 µg/ml	9.3	8.2
Fosamprenavir 7 µg/ml	21.8	17.7
Fosamprenavir 35 µg/ml	10.6	13.1
Atazanavir 0.6 µg/ml	7.2	6.4
Atazanavir 3 µg/ml	6.0	6.2
Atazanavir 15 µg/ml	4.3	5.1
Tipranavir 1 µg/ml	7.2	6.7
Tipranavir 5 µg/ml	9.2	9.1
Tipranavir 25 µg/ml	10.8	8.9
Darunavir 1.8 µg/ml	8.1	6.0
Darunavir 9 µg/ml	8.6	5.9
Darunavir 45 µg/ml	4.9	5.5

DISCUSSION

The biological activity of OPG and RANKL plays an important role in bone metabolism control. Impairment of the OPG/RANKL ratio may affect the homeostasis of bone structures (Matsuo and Irie 2008). RANKL, in association with M-CSF, regulates the differentiation and the osteoclast activity of bone resorption that takes place before the bone rebuilding mediated by osteoblasts, whereas OPG inhibits the RANKL/RANK interaction on target cell membranes negatively modulating the RANKL biological effects (Vaananem *et al.* 2008, Wright *et al.,* 2009). Since some recent papers (Mora *et al.* 2007, Gibellini *et al.* 2008) disclosed that these cytokines and the OPG/RANKL plasma ratio can be affected by HIV infection, we focused our attention on whether some antiretroviral compounds can regulate the RANKL and OPG content in the early treatment time in the surnatants of an osteoblast-like human cell line model represented by HOBIT cells. This cell model was chosen because the osteoblast is an important source of RANKL and OPG synthesis and it plays an essential role in bone mass control. The activity of antiretroviral molecules on bone structures is controversial: in recent years, several clinical papers have described opposite results regarding the correlation between osteopenia/osteoporosis and HAART (Cozzolino *et al.,* 2003). This discrepancy is probably due to different approaches in the selection of patients, analysis methods and treatments evidenced in these reports. However, an in-depth meta-analysis (Brown and Qaqish 2006) suggested a relationship between protease inhibitor (PI) treatment and bone mass loss even though the mechanisms and the true role of each PI has not yet been addressed. Some studies performed on murine models showed that only some PIs such as nelfinavir, saquinavir, indinavir and ritonavir increase the bone resorption activity whereas lopinavir and amprenavir did not induce any bone-related effect (Jain and Lenhard 2002). Saquinavir is able to interfere with TRAF-6 degradation, an important factor involved in the negative regulation of the differentiation and survival of osteoclasts (Fakruddin and Laurence 2003). A few studies have been performed on the relationship between PIs and osteoblasts. It is noteworthy that indinavir but not the saquinavir decreased the osteoblast activity through the reduction of alkaline phosphatase function, calcium deposition and RUNX-2 expression (Malizia *et al.,* 2007). In addition, some PIs inhibited vitamin D metabolism and hence may impair the bone mass. In fact, Indinavir negatively affected the 1α-hydroxylase enzyme and, to a lesser extent, 25-hydroxylase enzyme activity reducing 1,25-dihydroxyvitamin D3 production (Dusso *et al.,* 2000, Cozzolino *et al.,* 2003).

In this report, the effects of specific PIs on RANKL and OPG yielding were analyzed in an osteoblast-like cell line model. The data obtained in the early treatment time (24-48 hours) showed some positive or negative variations only when optimal (C2) and/or higher concentrations (C3) of specific PIs were used. In particular we focused our attention on the action of single drugS such as saquinavir, atazanavir and indinavir, fosamprenavir and darunavir, ruling out lopinavir, usually administrated as a coformulation (lopinavir/ritonavir).

Saquinavir, atazanavir and indinavir did not exhibit any effect on RANKL or OPG content in

HOBIT surnatants in any of the tested experimental conditions. Therefore, fosamprenavir can induce a significant increase in OPG coupled with a decrease of RANKL using C2 concentration determining a consistent increase of OPG/RANKL ratio (21.8 vs 6.9 at 24 hours and 17.7 vs 6.4 at 48 hours). Interestingly, fosamprenavir used at C3 concentration confirmed a significant increase in OPG whereas the decrease of RANKL was not significant. These data suggest that C2 is optimal to determine RANKL decrease, while C3 may activate or inhibit specific intracellular pathways interfering with the RANKL decrease. This phenomenon is well known in scientific literature: for example a molecule can induce specific biological effects at optimal concentration but the progressive concentration increase of treatment may determine an impairment of biological signals such as the squelching effect with the possible appearance of opposite biologic effects (Levine and Manley 1989, Barillari and Ensoli 2002). Hence, fosamprenavir at C2 concentration has, in the early treatment time, a possible positive effect on the anabolic bone function of osteoblasts.

Tipranavir induced the decrease of RANKL and OPG content with a slight increase in the OPG/RANKL ratio when C2 or C3 concentrations were used. It is noteworthy that tipranavir, but not all the other PIs tested, may induce a small, but significant apoptosis activation at C3 concentration. Hence, when the treatment was performed using tipranavir at C3 concentration it is possible that the decrease of RANKL and OPG may be related to pro-apoptotic induction by tipranavir and not only to putative specific mechanisms. The analysis of the early effects of tipranavir at C2 concentration suggested that the concomitant decrease of RANKL and OPG with a relative increase in the OPG/RANKL ratio probably do not interfere with bone homeostasis. By contrast, tipranavir might play a negative role at higher concentrations because it may potentially induce apoptosis activation in the osteoblasts.

Darunavir exhibited a specific increase in RANKL at 24 hours at C3 with a slight decrease of the OPG/RANKL ratio. This RANKL activation is a transient effect but it is interesting because it suggests that over-optimal darunavir concentrations may induce an increase in RANKL and then a hypothetical induction of osteoclast activity and bone resorption especially in chronic treatment. Further studies are in progress to investigate a possible correlation of different levels of RANKL and OPG and some protease inhibitors, since a modified balance of the bone remodeling-unit could be linked with a variation of bone turnover, assessed by the detection and the meaning of markers of bone formation and bone reabsorption.

In conclusion, this report showed that only specific PIs are involved in the perturbation of OPG/RANKL system at early treatment time in an osteoblast-like cell line. This model is useful because it allows the analysis of one of the most important biological pathways regulating bone homeostasis in specific cell lines treated by specific PIs. On the other hand, this analysis is not exhaustive because it cannot describe the effects on osteoblasts of treatment with an association of antiretrovirals or the adjunctive possible interaction with HIV-related inflammatory cytokines that could affect OPG/RANKL synthesis. In addition, this study should be enlarged to include osteoclasts to investigate pivotal osteoblast/osteoclast cross-talk.

This paper, therefore, is the first step to dissect the relationship between specific PIs and osteoblasts regarding bone mass impairment to identify and evaluate new aspects to monitor during the HAART therapy in the HIV-infected patients.

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REFERENCES

Baldini G., Ponti C., Bortul R., Narducci P., Grill V., Martelli A.M. (2008). Sparc localizes to the blebs of hobit cells and human primary osteoblasts. *J. Cell. Biochem.* **104**, 2310-2323.

Barillari G., Ensoli E. (2002). Angiogenic effects of extracellular human immunodeficiency virus type 1 Tat protein and its role in the pathogenesis of AIDS-associated Kaposi's sarcoma. *Clin. Microbiol. Rev.* **15**, 310-326.

Borderi M., Gibellini D., Vescini F., De Crignis E., Cimatti L., Biagetti C., Tampellini L., Re M.C. Metabolic bone disease in HIV infection. (2009). *AIDS*. **23**, 1297-1310.

Brown T.T., Qaqish R.B. (2006). Antiretroviral therapy and the prevalence of osteopenia and osteoporosis: a meta-analytic review. *AIDS*. **20**, 2165-2174.

Cozzolino M., Vidal M., Arcidiacono M.V., Tebas P., Yarasheski K.E., Dusso A.S. (2003). HIV-protease inhibitors impair vitamin D bioactivation to 1,25-dihydroxyvitamin D. *AIDS*. **17**, 513-520.

De Crignis E., Cimatti L., Borderi M., Gibellini D., Re M.C. (2008). Bone alterations during HIV infection. *New Microbiol.* **31**, 155-164.

Dusso A., Vidal M., Powderly W.G., Yarasheski K.E., Tebas P. (2000) Protease inhibitors inhibit in vitro conversion of 25(OH)-vitamin D to 1,25 (OH)2-vitamin D. *Antiviral Therapy*. **6**, 10-18.

Electronic Medicines Compendium. http://emc.medicines.org.uk/

Fakruddin J.M., Laurence J.J. (2003). HIV envelope gp120-mediated regulation of osteoclastogenesis via receptor activator of nuclear factor kappa B ligand (RANKL) secretion and its modulation by certain HIV protease inhibitors through interferon-gamma/RANKL cross-talk. *Biol. Chem.* **278**, 48251-48258.

Fauci A.S. (2003). HIV and AIDS: 20 years of science. *Nat. Med*. **9**, 839-843.

Gibellini D., Borderi M., De Crignis E., Cicola R., Vescini F., Caudarella R., Chiodo F., Re M.C. (2007). RANKL/OPG/TRAIL plasma levels and bone mass loss evaluation in antiretroviral naive HIV-1-positive men. *J. Med. Virol.* **79**, 446-1454.

Gibellini D., De Crignis E., Ponti C., Cimatti L., Borderi M., Tschon M., Giardino R., Re M.C. (2008). HIV-1 triggers apoptosis in primary osteoblasts and HOBIT cells through TNFalpha activation. *J. Med. Virol.* **80**, 1507-1514.

Haggerty C.M., Pitt E., Siliciano R.F. (2006). The latent reservoir for HIV-1 in resting CD4+ T cells and other viral reservoirs during chronic infection: insights from treatment and treatment-interruption trials. *Curr. Opin. HIV. AIDS.* **1**, 62-69.

Jain R.G., Lenhard J.M. (2002). Select HIV protease inhibitors alter bone and fat metabolism ex vivo. *J. Biol. Chem.* **277**, 19247-19250.

Konishi M., Takahashi K., Yoshimoto E., Uno K., Kasahara K., Mikasa K. (2005). Association between osteopenia/osteoporosis and the serum RANKL in HIV-infected patients. *AIDS.* **19**, 1240-1241.

Levine M., Manley J.L. (1989). Transcriptional repression of eukaryotic promoters. *Cell.* **59**, 405-409.

Levy J.A. (2009). HIV pathogenesis: 25 years of progress and persistent challenges. *AIDS.* **23**, 147-160.

Malizia A.P., Cotter E, Chew N, Powderly W.G, Doran P.P. (2007). HIV protease inhibitors selectively induce gene expression alterations associated with reduced calcium deposition in primary human osteoblasts. *AIDS Res. Hum. Retroviruses.* 23: 243-250.

Marie P.J. (2008). Transcription factors controlling osteoblastogenesis. *Arch. Biochem. Biophys.* **473**, 201-209.

Matsuo K., Irie N. (2008). Osteoclast-osteoblast communication. *Arch. Biochem. Bioph.* **473**, 201-209.

Mondy K., Tebas P. (2003). Emerging bone problems in patients infected with human immunodeficiency virus. *Clin. Infect. Dis.* **36**, S101-105.

Mora S., Zamproni I., Cafarelli L., Giacomet V., Erba P., Zuccotti G., Viganò A. (2007). Alterations in circulating osteoimmune factors may be responsible for high bone resorption rate in HIV-infected children and adolescents. *AIDS.* **21**, 1129-1135.

Nacher M., Serrano S., González A., Hernández A., Marinoso M.L., Vilella R., Hinarejos P., Díez A., Aubia J. (2001). Osteoblasts in HIV-infected patients: HIV-1 infection and cell function. *AIDS.* **15**, 2239-2243.

Re M.C., Vitone F., Bon I., Schiavone P., Gibellini D. (2006). Meaning of DNA detection during the follow-up of HIV-1 infected patients: a brief review. *New Microbiol.* **29**, 81-8 8.

Serrano S., Marinoso M.L., Soriano J.C., Rubiés-Prat J., Aubia J., Coll J., Bosch J., Del Rio L., Vila J., Goday A. (1995). Bone remodelling in human immunodeficiency virus-1-infected patients. A histomorphometric study. *Bone.* **16**, 185-191.

Vaanänen H.K., Laitala-Leinonen T. (2008). Osteoclast lineage and function. *Arch. Biochem. Biophys.* **8**, 473, 132-138.

Wright E.J. (2009). Neurological disease: the effects of HIV and antiretroviral therapy and the implications for early antiretroviral therapy initiation. *Curr. Rev. Musculoskelet. Med.* **2**, 56-64.