

Inhibition of osteoblast differentiation by ritonavir

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Abstract. Osteoporosis is one of the chronic complications seen in human immunodeficiency virus (HIV)-infected patients, and affects patients at high prevalence. The causes of osteoporosis in HIV-infected patients are multiple, and include chronic HIV infection, living habits such as smoking and alcohol consumption, and antiretroviral drug use. Among antiretroviral drugs, protease inhibitors have been reported to be associated with osteoporosis. However, it remains to be determined how anti-HIV drugs affect osteoblast differentiation. In the current study, MC3T3-E1 cells, a mouse osteoblastic cell line, were cultured in osteoblast differentiation medium with or without different protease inhibitors (ritonavir, lopinavir, darunavir or atazanavir), and alkaline phosphatase (ALP) activity and the expression of Runt-related transcription factor 2 (Runx2) were analyzed. The ALP activity in MC3T3-E1 cells cultured with ritonavir was significantly reduced compared with that in cells in only osteoblast inducer reagent, indicating that ritonavir inhibited osteoblast differentiation. Meanwhile, ALP activity was not reduced in cells cultured with any of the other inhibitors. In addition, ritonavir inhibited the expression of Runx2, a key regulator of osteoblast differentiation, in the early period of osteoblast differentiation. To the best of our knowledge, this is the first study to demonstrate that ritonavir inhibits osteoblast differentiation *in vitro*. The present findings may explain the mechanism of osteopenia induced by combination antiretroviral therapy involving protease inhibitors.

Introduction

The progress of combination antiretroviral therapy (cART) has led to increased life expectancy of patients infected with human immunodeficiency virus (HIV) (1). However, as life expectancy has increased, chronic complications have become key chal-

lenges (2). Osteoporosis is among the chronic complications seen in HIV-infected patients; the prevalence of osteoporosis is reportedly three times higher among HIV-infected patients than non-HIV-infected patients (3). The reported prevalence rate of osteopenia in HIV-infected cohorts ranges from 22 to 71%, and that of osteoporosis from 3 to 33% (4). The causes of osteoporosis in HIV-infected patients are multiple, and include chronic HIV infection, living habits such as smoking and alcohol consumption, and antiretroviral drug use (5). Among antiretroviral drugs, protease inhibitors have been reported to be associated with osteoporosis, and antiretroviral regimens containing protease inhibitors can accelerate osteopenia and osteoporosis (6).

Osteoporosis is caused by an imbalance of bone resorption and formation (7). Osteoclasts have a role in bone formation, and osteoblasts that are derived from mesenchymal stem cells are responsible for bone formation (8). Osteoblast differentiation is regulated by transcription factors including Runt-related transcription factor 2 (Runx2) (9). Runx2 is a positive regulator of genes associated with bone matrix proteins including collagen type I α 1/2 chain (9), and triggers the expression of major bone matrix genes during the early stages of osteoblast differentiation (9). As such, Runx2 is considered to serve a central role in skeletal development and be associated with osteoporosis (9). However, it remains to be determined specifically how anti-HIV drugs affect osteoblast differentiation. Thus, in the present study, the influence of anti-HIV drugs on osteoblast differentiation was examined *in vitro*.

Materials and methods

Cell line and culture. The clonal mouse osteoblastic cell line, MC3T3-E1 subclone 14, was purchased from American Type Culture Collection (Manassas, VA, USA). The MC3T3-E1 cells were cultured at 37°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% fetal bovine serum (Hyclone; GE Healthcare Life Science, Logan, UT, USA), 100 IU/ml penicillin and 100 μ g/ml streptomycin. To induce osteoblast differentiation, MC3T3-E1 cells were cultured at 37°C in 5% CO₂ in DMEM with osteoblast inducer reagent containing 1% L-ascorbic acid, 2% β -glycerophosphate and 0.2% hydrocortisone (Takara Bio, Inc., Otsu, Japan). The medium was changed every other day.

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Anti-HIV drugs. The HIV protease inhibitors ritonavir, darunavir, atazanavir and lopinavir were purchased from Toronto Research Chemicals, Inc. (Toronto, ON, Canada). These drugs were prepared as stock solutions in methanol. All prepared anti-HIV drugs were mixed with osteoblast differentiation medium to adjust to the maximum serum concentration (Cmax) that these drugs achieve when they are administered at treatment doses in adult HIV patients. Cmax values of protease inhibitors were based on those on the University of Liverpool website, www.hiv-druginteraction.org. The Cmax values were: Ritonavir, 11.20 µg/ml; lopinavir, 9.60 µg/ml; darunavir, 6.50 µg/ml; and atazanavir 3.15 µg/ml.

Alkaline phosphatase (ALP) activity. MC3T3-E1 cells were seeded at a density of 1×10^4 cells/well in a 96-well microplate in normal culture medium. To induce differentiation, the medium was replaced by DMEM with osteoblast inducer reagent after 1 day in the presence or absence of each protease inhibitor, respectively. The cells were incubated for 7 and 9 days in four types of medium: i) DMEM; ii) DMEM with osteoblast inducer reagent; iii) DMEM with osteoblast inducer reagent and an anti-HIV drug; and iv) DMEM with vehicle (methanol). For subsequent treatments with ritonavir, MC3T3-E1 cells were treated with various concentrations (0.1, 0.5, 1.0, 5.0 and 10.0 µg/ml ritonavir) and for various durations (10.0 µg/ml ritonavir for 9, 11 and 14 days). All cells were cultured at 37°C in a 5% CO₂ atmosphere. ALP activity was evaluated using a TRACP & ALP double-staining kit (Takara Bio, Inc.) according to the manufacturer's protocol. Briefly, the plates were incubated with kit reagents at 37°C for 15 min, absorbance was measured at 405 nm, and ALP activity was calculated from a standard value (bone ALP; Takara Bio, Inc.). The total protein concentration of each cell was determined by a DC™ Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the activity data were normalized to the total protein concentration.

Alizarin Red staining. MC3T3-E1 cells were seeded at a density of 4×10^4 cells/well in a 24-well plate. The cells were incubated with 10.0 µg/ml ritonavir or control mediums as above. Mineralization of MC3T3-E1 cells was confirmed by using an Alizarin Red S staining kit (Cosmo Bio, Co., Ltd., Tokyo, Japan) after 7, 14, 21 and 28 days of culture. Then, cells were washed three times with phosphate-buffered saline, with 500 µl of 100% methanol added to each well, and incubated at 4°C for 20 min. Alizarin Red solution was added to each well and incubated at room temperature for 5 min. Each well was then washed again three times and directly observed.

Total RNA extraction and cDNA synthesis. MC3T3-E1 cells seeded at 1×10^5 cells/well in a 12-well plate were incubated with 10.0 µg/ml ritonavir or control mediums as above for 3, 5 and 7 days. Total RNA was extracted from the MC3T3-E1 cells by adding 350 µl Tripure (Roche, Basel, Switzerland) to each well. RNA was isolated according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA by using ReverTra Ace® qPCR RT Master Mix (Toyobo Life Science, Osaka, Japan) according to the manufacturer's instructions.

Quantitative polymerase chain reaction (qPCR). Runx2 gene expression in MC3T3-E1 cells was examined by qPCR. β-actin was used as an internal control gene. The qPCR was performed with a Roche LightCycler 480 system using a LightCycler 480 Probe Master kit (Roche Diagnostics, Basel, Switzerland). Reactions were performed in a final total volume of 20 µl containing 1x LightCycler 480 Probe Master mix, 0.1 µM of each forward and reverse primer, 3.8 µl distilled H₂O and 5 µl of the cDNA template. Specific primers for mouse Runx2 and β-actin were designed using the Universal ProbeLibrary (Roche Diagnostics). The primers used in the current study were as follows: For Runx2, forward, 5'-cgtgtcagcaaagctctctttt-3' and reverse, 5'-ggctcagctgcctcatct-3'; and for β-actin, forward, 5'-tgacaggatgcagaaggaga-3' and reverse, 5'-cgctcaggaggagcaatg-3'. The amplification conditions were as follows: Denaturation at 95°C for 5 min, 45 cycles of amplification (95°C for 10 sec, 60°C for 30 sec and 72°C for 1 sec), and cooling at 50°C for 10 sec. Relative quantification of target gene expression was determined using the E-Method (Efficiency Method) from the LightCycler 480 software (10).

Lactate dehydrogenase (LDH) activity assay. LDH activity was measured to determine MC3T3-E1 cell viability following treatment with ritonavir. MC3T3-E1 cells were plated in 12-well plates (1×10^5 cells/well) and incubated in DMEM with osteoblast inducer reagent in the presence or absence of ritonavir (10 µg/ml). On day 8, culture medium was replenished and cells were cultured again for 24 h. Cell medium and lysate extracted with radioimmunoprecipitation assay buffer (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were collected on day 9, and cell viability was evaluated using Cytotoxicity detection KitPLUS (Roche Diagnostics) according to the manufacturer's protocol. Cells in DMEM only were defined as a control. The absorbance was measured at 490 nm, and cell viability was calculated using the following formula: Cell viability (%) = $\{1 - [(absorbance\ of\ medium - absorbance\ of\ control\ medium) / (absorbance\ of\ lysate - absorbance\ of\ control\ lysate)]\} \times 100$.

Statistical analysis. Significant differences among samples were determined by one-way analysis of variance with post hoc Tukey's honest significant difference test, with $P < 0.05$ considered to indicate statistical significance. At least three samples were tested in three independent experiments. All results are presented as means ± standard error of the mean. The data analysis was performed using JMP software version 12.2 (SAS Institute, Inc., Cary, NC, USA).

Results

Osteoblast differentiation is inhibited by ritonavir. As documented, osteoblasts are differentiated by a medium that contains L-ascorbic acid, β-glycerophosphate and steroid (11). Since ALP activity is enhanced during osteoblast differentiation *in vitro*, ALP may be used as an osteogenic differentiation marker (12). First, the present study observed that ALP activity was enhanced in MC3T3-E1 cells at 7 days after the addition of DMEM with osteoblast inducer reagent compared with in cells cultured in DMEM only ($P < 0.05$; data not shown). Subsequently, the effect of protease inhibitors on

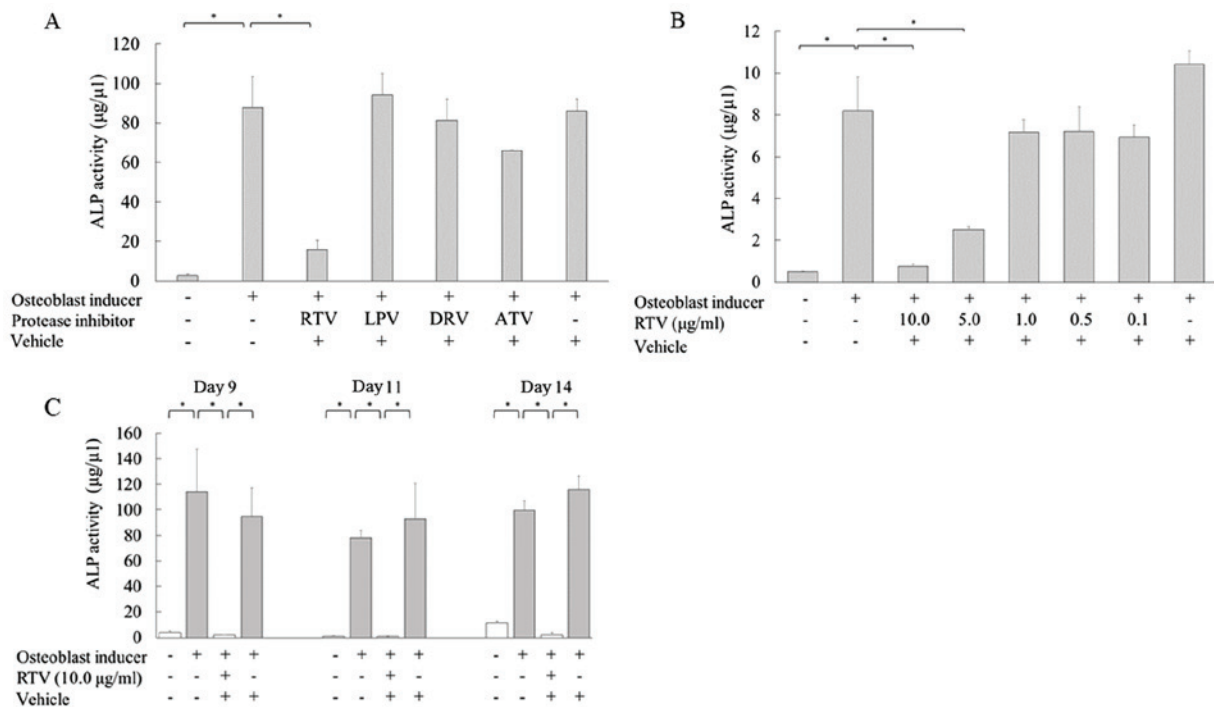


Figure 1. Ritonavir reduces the ALP activity in MC3T3-E1 cells. (A) MC3T3-E1 cells were cultured in osteoblast differentiation medium with or without Cmax doses of protease inhibitors for 9 days, and ALP activity was analyzed. (B) Cells were cultured in osteoblast differentiation medium with various concentrations of ritonavir (0, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/ml) for 9 days, and ALP activity was measured quantitatively. (C) Cells were cultured in osteoblast differentiation medium with or without 10.0 µg/ml ritonavir for 9, 11 and 14 days, and ALP activity was analyzed. All data are presented as means ± standard deviation. *P<0.05. ALP, alkaline phosphatase; Cmax, maximum serum concentration; RTV, ritonavir; LPV, lopinavir; DRV, darunavir; ATV, atazanavir.

osteoblast differentiation was examined. MC3T3-E1 cells were cultured in osteogenic differentiation medium in the presence or absence of the protease inhibitors ritonavir, lopinavir, darunavir and atazanavir 3.15 µg/ml at their Cmax for 9 days, and then ALP activity was examined. The ALP activity of MC3T3-E1 cells cultured with ritonavir was significantly reduced compared with that of the control cells in osteoblast induction medium alone (P<0.05). However, ALP activity was not reduced in cells cultured with the other anti-HIV drugs (Fig. 1A). These results indicated that the protease inhibitor ritonavir inhibited osteoblast differentiation.

Next, the relationship between ritonavir concentration and osteoblast differentiation was examined. MC3T3-E1 cells were treated with various concentrations of ritonavir (0.1, 0.5, 1.0, 5.0 and 10.0 µg/ml). ALP activity was significantly reduced in MC3T3-E1 cells cultured with 5.0 or 10.0 µg/ml ritonavir compared with the controls in osteoblast induction medium alone (P<0.05); however, lower concentrations of ritonavir did not inhibit ALP activity (Fig. 1B).

The time course of ALP activity in MC3T3-E1 cells cultured with ritonavir was also examined. MC3T3-E1 cells were cultured in osteogenic differentiation medium with or without 10.0 µg/ml ritonavir for 9, 11 and 14 days. ALP activity was significantly reduced following ritonavir treatment at each time point (P<0.05; Fig. 1C).

Effect of ritonavir on the expression of Runx2 in MC3T3-E1 cells. RUNX2 is the key gene responsible for the differentiation of human mesenchymal cells into osteoblasts; it upregulates bone matrix proteins including ALP in the early phase of osteoblast differentiation (13). MC3T3-E1 cells were cultured in

osteogenic differentiation medium with or without 10.0 µg/ml ritonavir for 3, 5 and 7 days, and the mRNA expression of Runx2 was measured by reverse transcription-qPCR. On days 3 and 5, the mRNA levels of Runx2 were significantly reduced in differentiating cells cultured with ritonavir compared with in the differentiating controls (P<0.05); however, no significant differences were observed on day 7 (Fig. 2). These results indicated that ritonavir inhibited expression of Runx2 in the early phase of osteoblast differentiation.

Osteoblast mineralization is inhibited by ritonavir. In the process of bone formation, osteoblasts need to differentiate and mineralize. Thus, whether ritonavir inhibited osteoblast mineralization was examined. MC3T3-E1 cells were cultured in DMEM with osteoblast inducer reagent with or without 10 µg/ml ritonavir for 7, 14, 21 and 28 days. Then, cells were stained with Alizarin Red S to assess mineralization. At 7 and 14 days, cells treated with DMEM or with osteoblast induction medium, with or without ritonavir, exhibited no positive staining indicative of mineralization. At 21 and 28 days, cells treated with osteoblast induction medium with or without vehicle exhibited increasing mineralization, while those treated with DMEM only or osteoblast induction medium with ritonavir were not (Fig. 3). These results indicated that ritonavir suppressed or delayed osteoblast mineralization.

Cell viability of MC3T3-E1 cells treated with ritonavir. The cytotoxicity of ritonavir was assessed via measuring LDH activity. MC3T3-E1 cells were cultured in osteogenic differentiation medium with or without 10.0 µg/ml ritonavir for 9 days, and the levels of LDH activity were measured.

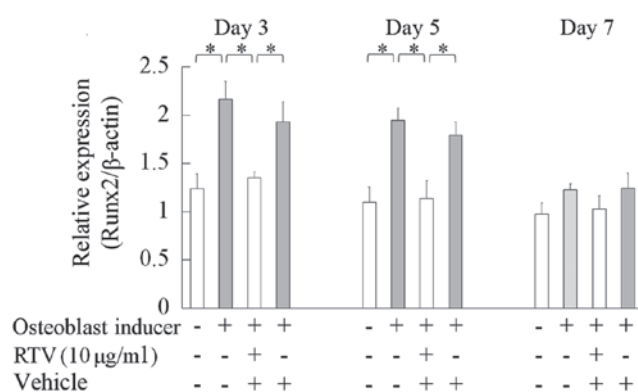


Figure 2. Ritonavir reduces the expression of Runx2 mRNA in MC3T3-E1 cells. Cells were cultured in osteoblast differentiation medium with or without 10.0 μg/ml ritonavir for 3, 5 and 7 days. Runx2 mRNA expression was evaluated by reverse transcription-quantitative polymerase chain reaction. The data are presented as means ± standard deviation. *P<0.05. Runx2, runt-related transcription factor 2; RTV, ritonavir.

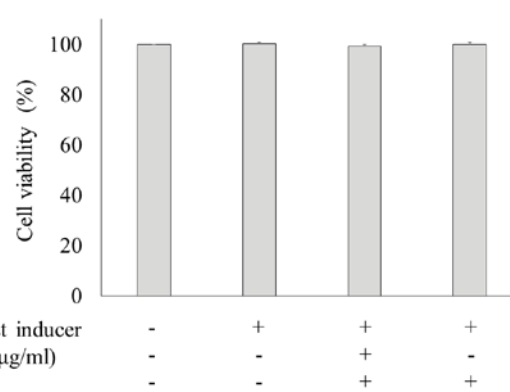


Figure 4. Ritonavir did not affect MC3T3-E1 cell viability. Cells were cultured in osteoblast differentiation medium with or without 10.0 μg/ml ritonavir for 9 days, and the levels of LDH activity were measured. The data are presented as means ± standard deviation. LDH, lactate dehydrogenase; RTV, ritonavir.

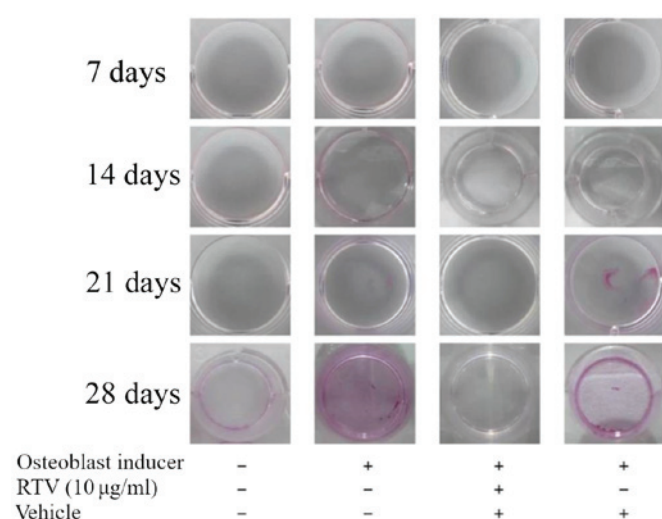


Figure 3. Ritonavir suppresses osteoblast mineralization in MC3T3-E1 cells. Cells were cultured in osteoblast differentiation medium with or without 10.0 μg/ml ritonavir for up to 28 days. Alizarin red S staining was performed. RTV, ritonavir.

There were no statistically significant differences among the groups (Fig. 4). These results showed that ritonavir did not exert cytotoxic effects in MC3T3-E1 cells.

Discussion

Osteoporosis is one of the complications seen in patients with HIV. HIV itself suppresses osteogenesis (14). Several *in vivo* studies have reported that osteopenia and osteoporosis are common among patients treated with antiretroviral drugs (15-18). Bedimo *et al* (15) reported that cumulative exposure to tenofovir and protease inhibitors, particularly lopinavir/ritonavir, was an independent predictor of increased risk of osteoporotic fracture in HIV patients on cART. Further, it was shown in the Data collection on Adverse events of anti-HIV Drugs study that protease inhibitor use was one of the risk factors for osteoporosis (16,17). Duvivier *et al* (18) reported that decreased lumbar spine bone mineral density

was more pronounced in patients receiving protease inhibitors (indinavir/ritonavir or lopinavir/ritonavir) compared with other antiretroviral drugs. By contrast, a cross-sectional study identified decreased bone density in HIV-positive patients irrespective of treatment with or without protease inhibitors (19). Thus, the association between osteoporosis and protease inhibitors *in vivo* is yet to be confirmed.

The MC3T3-E1 cell line is a pre-osteoblast line derived from mouse calvaria (20). It is established to exhibit a time-dependent and sequential expression of osteoblast characteristics analogous to *in vivo* bone formation, and is used as a bone differentiation and mineralization model *in vitro* (21). In the present study, ritonavir was indicated to affect the pathway of osteoblast differentiation and the time course of differentiation. There are a number of *in vitro* reports describing the relationship between certain antiretroviral drugs and osteoblast differentiation (21,22). Jain and Lenhard reported that two protease inhibitors, lopinavir and nelfinavir, decreased osteoblast ALP activity and gene expression in human mesenchymal stem cells (21). Another report demonstrated that ALP activity decreased significantly in human osteoblast cultures following exposure to nelfinavir and indinavir (22). Santiago *et al* has previously reported ritonavir may facilitate osteoclast differentiation (23); however, other findings have suggested that ritonavir could inhibit osteoclast formation and function (24). Thus, the exact effect of RTV on osteoblast cells remained unknown.

Ritonavir was originally used for its antiviral action, but is now used as a booster of other protease inhibitors. The therapeutic adult dose of ritonavir is 600 mg twice a day. When 600 mg ritonavir is taken twice a day, the mean maximum and minimum serum concentrations (C_{max} and C_{min}) of ritonavir have been determined as 11.2±3.6 and 3.7±2.6 μg/ml, respectively (25). In the present study, osteoblast differentiation was inhibited by 5.0 and 10.0 μg/ml ritonavir. These results indicated the possibility that administration of therapeutic doses of ritonavir may inhibit osteoblast differentiation *in vivo*. However, as a booster, ritonavir is used at a dose of 100 to 200 mg a day, and the expected ritonavir C_{max} is ~1.5 μg/ml, and therefore osteoblast differentiation appears unlikely to be inhibited *in vivo*. However, to our knowledge

there are no reports of a pharmacokinetic analysis of ritonavir in bone. Thus, osteoblast differentiation may be suppressed in bone when the booster dose of ritonavir is administered.

In the present study, ritonavir suppressed the expression of Runx2 mRNA. To the best of our knowledge, this is the first study to report a suppressive effect of ritonavir on the expression of Runx2, and that ritonavir may affect osteoblast differentiation and mineralization in MC3T3-E1 cells. There are numerous pathways associated with osteoblast differentiation including the bone morphogenetic protein BMP and parathyroid hormone pathways (26). Runx2 is an essential transcription factor required for osteogenesis; Runx2-knockout mice exhibit a complete absence of mature osteoblasts and ossification (26). In particular, Runx2 is a key regulator of osteoblast differentiation and regulates the expression of several osteoblastic genes, including collagen 1, osteopontin, osteocalcin and bone sialoprotein, to induce differentiation (13). In primary osteoblast cells isolated from the calvariae of rats, study has found ALP activity to be significantly decreased by Runx2 small interfering RNA treatment when cells were treated with osteoblast differentiation medium that included ascorbic acid and β -glycerophosphate (27), indicating that Runx2 expression is upstream of ALP. In the present study, the expression of Runx2 mRNA was suppressed on days 3 and 5, but not on day 7 of treatment with ritonavir in osteoblast differentiation medium. Prior to day 7, the ALP activities of cells with or without differentiation medium did not change (not shown). But ALP activities were suppressed on day 9 or later of treatment with ritonavir in osteoblast differentiation medium. These results collectively indicate that ritonavir suppressed Runx2 directly or other regulators of osteoblast differentiation upstream of Runx2, and, as a result, ALP activity was suppressed.

The present results also suggested inhibition of osteoblast mineralization by ritonavir. However, bone formation takes 3 to 4 months (28), and cells in the current study were cultured for only 28 days (4 weeks). Thus, the possibility that ritonavir simply delayed osteoblast mineralization cannot be excluded.

In conclusion, the present study is seemingly the first to demonstrate that ritonavir may inhibit osteoblast differentiation *in vitro*. Expression of Runx2 was suppressed by ritonavir in MC3T3-E1 cells. This may explain the mechanism of osteopenia induced by cART involving protease inhibitors.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW, YY, KS, IK, TK and YO designed the study. YW processed the experimental data, performed the analysis,

drafted the manuscript and produced the figures. All authors discussed the results and contributed to the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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