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11 β -hydroxysteroid dehydrogenase type 1 selective inhibitor BVT.2733 protects osteoblasts against endogenous glucocorticoid induced dysfunction

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Abstract. Pharmacologic glucocorticoids (GCs) inhibit osteoblast function and induce osteoporosis. 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) may play a role in osteoporosis as it regulates GC action at a pre-receptor level by converting inactive GC to its active form. Further, 11 β -HSD1 was found increasingly expressed in bone with age. In spite of these observations, its function in senile osteoporosis remains uncertain. In this study we constructed a lentiviral vector overexpressing mouse 11 β -HSD1 and then MC3T3-E1 preosteoblast cells were infected by the negative control lentivirus and 11 β -HSD1-overexpressing lentivirus, respectively. The mRNA and protein levels of 11 β -HSD1 were significantly increased in MC3T3-E1 cells that were infected by 11 β -HSD1-overexpressing lentivirus compared to the cells infected by the negative control lentivirus. The osteogenic differentiation of MC3T3-E1 preosteoblast cells was dramatically suppressed by 11 β -HSD1 overexpression under the reductase substrate dehydrocorticosterone (DHC). The inhibition effect was similar to the inhibition of osteogenesis by over-dose GCs, including ALP activity, the ultimate calcium nodule formation as well as the expression of the osteogenic genes such as ALP, BSP, OPN and OCN. However, with addition of BVT.2733, a selective inhibitor of 11 β -HSD1, all of the above osteogenic repression effects by 11 β -HSD1 overexpression were reversed. Furthermore, a GC receptor antagonist RU486 also showed the similar effect, preventing inhibition of osteogenesis by 11 β -HSD1 overexpression. These results demonstrated that the specific 11 β -HSD1 inhibitor BVT.2733 can reverse the suppression effect towards osteogenic differentiation in 11 β -HSD1 overexpressed MC3T3-E1 cells. Inhibition of 11 β -HSD1 can be a new therapeutic strategy for senile osteoporosis.

Key words: BVT.2733, 11 β -hydroxysteroid dehydrogenase type 1, Senile osteoporosis, MC3T3-E1 preosteoblast, Lentivirus

SENILE OSTEOPOROSIS, mainly caused by impaired bone formation, has become a common disease and is seriously harmful to human health. In spite of growing concerns about age-related bone loss, the pathogenetic mechanism remains unclear.

Long-term and high dose glucocorticoid (GC) ther-

apy leads to osteoporosis [1-4]. 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1) in osteoblasts primarily displays reductase activity and converts inactive glucocorticoid to active glucocorticoid, acting as an intracellular amplifier of active glucocorticoid [5]. This enzyme regulates glucocorticoid action at a pre-receptor level, and thus plays a critical role in glucocorticoid responses [6]. Recent studies have revealed that 11 β -HSD1 likely plays an essential role in the process of senile osteoporosis. In mouse and human osteoblasts, the expression and reductase activity of 11 β -HSD1 increase with age. Aging in C57BL/6 mice was associated with an increase in adrenal production of glucocorticoids as well as induction of 11 β -HSD1 in bone tissue [7]. In the primary cultures of human

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osteoblasts, 11 β -HSD1 reductase activity was correlated positively with age, suggesting that the enhancement of 11 β -HSD1 reductase activity may play a critical role in age-related bone loss [13].

In spite of the key role of 11 β -HSD1 in regulating GC activity in specific tissues, most investigations of 11 β -HSD1 were focused on the outcome in treating metabolic syndrome by inhibiting 11 β -HSD1 activity. The earliest study by Kotelevtsev *et al.* showed that knocking out of 11 β -HSD1 gene in mice did result in attenuated glucocorticoid-inducible responses and resisted hyperglycemia on obesity [14]. There was only one report indicating that 11 β -HSD1 deficient mice were used to investigate the role of the intracrine activation of GCs on normal bone physiology *in vivo* [15]. 11 β -HSD1^{-/-} young mice exhibited no significant changes in cortical or trabecular bone mass compared with wild-type mice. However, there was no similar report in old mice. Thus, new knowledge is needed elucidating the response of 11 β -HSD1 regulation in bone metabolism of senile animals.

Another approach to regulate intracellular GC is mediated by transgenic expression of 11 β -HSD2 in mice, which exerts dehydrogenase activity by converting active GC to inactive forms [16]. 11 β -HSD2 is highly expressed in aldosterone target tissues such as the kidney but is not expressed in adult bone tissue. Through cell-specific overexpression of 11 β -HSD2, mice were protected from the adverse effects of aging on osteoblast and osteocyte apoptosis, bone formation rate and microarchitecture, in which osteoblasts and osteocytes were shielded from GCs [7]. The effectiveness of 11 β -HSD2 overexpression highlighted that specific inhibition of the 11 β -HSD1 reductase activity might be a new therapeutic strategy for treating of senile osteoporosis.

BVT.2733 is a small molecule that can inhibit mouse 11 β -HSD1 in an isoform-selective manner. Many research papers have confirmed that this molecule is an antagonist of 11 β -HSD1 but not 11 β -HSD2 [8-11]. In this research, BVT.2733 was synthesized by China Pharmaceutical University according to the patent information. In order to confirm BVT.2733 as a selective 11 β -HSD1 antagonist, we performed the GRE-Luc reporter experiment. As shown in Supplementary Fig. 1, inactive glucocorticoid DHC induced up-regulated luciferase activity through being converted into active glucocorticoid in the presence of endogenous 11 β -HSD1 activity. However, BVT.2733 reversed this

effect, confirming that BVT.2733 is a selective 11 β -HSD1 inhibitor.

The aim of the present study was to examine the effect of BVT.2733, a selective inhibitor of 11 β -HSD1 on endogenous GC induced dysfunction of MC3T3-E1 preosteoblasts. Because of poor transfection efficiency using lipid-dependent transfection methods in MC3T3-E1 preosteoblasts, we adapted the lentivirus mediated transfection as an alternate approach to construct a GC damaged cell model and to study the effect of 11 β -HSD1 inhibitor. Our data confirmed the notion that 11 β -HSD1 may be a very promising therapeutic target for senile osteoporosis.

Materials and Methods

Reagents

BVT.2733, an 11 β -HSD1 selective inhibitor was synthesized by China Pharmaceutical University according to the patent information. M-MLV, dNTP, RNase inhibitor and other reverse transcription reagents were purchased from Promega Corp (Madison, WI, USA). Anti-mouse 11 β -HSD1 and tubulin antibodies were purchased from R&D System. Trizol were purchased from Invitrogen (Carlsbad, CA, USA). Unless otherwise stated, all chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO), and cell culture materials were obtained from Invitrogen (Carlsbad, CA, USA).

Lentivirus preparation and infection of MC3T3 preosteoblasts

Full-length mouse cDNA sequence for 11 β -HSD1 was cloned into pLL3.7-BSD lentiviral vector. Recombinant lentiviruses were produced by cotransfecting 293T cells with the lentivirus expression plasmid and packaging plasmids using lipofectamine2000 (Invitrogen). Lentivirus particles were harvested 48 h after transfection and were filtered through 0.45 μ m cellulose acetate filters. Lentivirus solution was purified and concentrated in 5 % PEG8000 and 0.15 M NaCl for 16 h at 4 °C. The final lentivirus solution was obtained by centrifugation at 3000 g for 30 min and resuspension of the pellet in PBS. Lentiviral particles produced from empty expression vector were used as control. MC3T3-L1 preosteoblasts at 70 % confluence at low passages were infected with control and 11 β -HSD1 lentiviral particles. 12 hours after the infection, the medium was changed. Infected cells were selected with 1 μ g/mL of blasticidin (BSD, Invitrogen) for 6

days and used for the following experiments.

Cell culture

Mouse fibroblast MC3T3-L1 preosteoblast cell line was purchased from the institute of Biochemistry and cell Biology (Shanghai, china). The primary osteoblasts were obtained from 5-7 day newborn mouse calvariae using sequential digestion by collagenase and trypsin [17]. Cells were maintained in α -MEM (Invitrogen, Carlsbad, CA) culture medium containing 10 % fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin. Cells were incubated at 37°C in a 5% CO₂ humidified atmosphere, and the medium was replaced every 3 days.

Luciferase assay

The Glucocorticoid response-luciferase plasmid (pGRE-luc) was purchased from Beyotime Institute of Biotechnology, China. Transient transfection was performed with Lipofectamine 2000 (Invitrogen) in 3T3-L1 fibroblasts. Luciferase activities were assayed 24 h after transfection using a luciferase reporter assay system (Promega).

Osteogenic differentiation

Cells were plated in 12-well plates at a density of 4×10^5 cells/well. 2 days after cells reached complete confluence, the medium was replaced with osteogenic medium (the above-mentioned media supplemented with 50 μ g/mL ascorbic acid and 10 mM β -glycerolphosphate). Medium were changed every 3 days and cells were induced for 14 days until mineralization occurred thoroughly.

For alkaline phosphatase (ALP) activity assays, cells were collected after induction of 6 days. Briefly, cells were washed with PBS and lysed in the lysis buffer (250 mM NaCl, 0.1% NP40, 50 mM HEPES, pH7.5). The lysate was incubated with p-nitrophenyl substrate

according to manufacturer's instructions (Sigma-aldrich). ALP activity was normalized to total protein. The protein concentration of cell lysates was determined with a BCA Protein Assay Reagent kit (Pierce).

Mineralization assay was performed after 14 days. Cells were rinsed with PBS, fixed with 4 % paraformaldehyde for 10 min and added with 1 % alizarin red (Sigma) for another 10 min. Nonspecific staining was removed by several wash with water. The staining was extracted from the cell matrix by incubation with 10 % cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 10 min. The alizarin red concentration was determined by measuring the absorbance at 550 nm. Results were expressed as the relative value compared to controls.

RNA preparation and quantitative real-time PCR

Total RNA was extracted from cultured cells using TRIZOL, followed by preparation of cDNA using the reverse transcription reagents. Quantitative real-time PCR was performed in triplicate with 0.2 μ M primers, 1 μ L cDNA and the SYBR Green Real-time PCR Master Mix (Roche Applied Science) in a total volume of 20 μ L. Reactions were run at 95°C for 60s, followed by 40 cycles of 15s at 95 °C, 15 s at 60 °C and 45s at 72 °C. The StepOne Plus Real-time PCR system from ABI was used for analysis. Results were expressed as cycle threshold (Ct) and calculated as Δ Ct, which were normalized to endogenous control 18S rRNA. Primer sequences were described in Table 1.

Western blotting

The protein levels of 11 β -HSD1 were detected by Western blotting. Briefly, Cultured cells were lysed using RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 % NP40, 0.5 % sodium deoxycholate, 5 mM EDTA, 5 mM NaF, 1 mM PMSF, 5 μ g/mL leupeptine and 5 μ g/mL aprotinin). Whole-cell lysates

Table 1 Primer sequences for real-time PCR

Gene	Forward primer*	Reverse primer
18S	CATGATTAAGAGGGACGGC	TTCAGCTTTGCAACCATACTC
11 β -HSD1	CAGAAATGCTCCAGGGAAAGAA	GCAGTCAATACCACATGGGC
ALP	CCAGCAGGTTTCTCTCTTGG	CTGGGAGTCTCATCCTGAGC
BSP	CAGAAGTGGATGAAAACGAG	CGGTGGCGAGGTGGTCCCAT
OPN	TATGCTCCGACCTCCTCAAC	AATAGGATTGGGAAGCAGAAAG
OCN	CTCTGCTCACTCTGCTGG	GCGTTGTAGGCGGTCTT

*Forward (F) and Reverse (R) primers were given for each gene at 5' to 3' direction as specified.

Fig. 1 Lentivirus provides efficient delivery of 11β-HSD1 genes into MC3T3-E1 preosteoblasts.

MC3T3-E1 preosteoblasts were infected with the 11β-HSD1 encoding lentivirus or nonencoding control lentivirus. After selection by blasticidin, total RNA or proteins of cells were collected for mRNA analysis or protein analysis of 11β-HSD1 expression. (A) Real time RT-PCR analysis of 11β-HSD1 mRNA expression. The mRNA values were normalized to the amounts of 18S rRNA. (B) Western blotting analysis of 11β-HSD1 expression. Tubulin was served as a loading control. Data are presented as means±SD, and results are representative of three independent experiments. ** $p < 0.01$ vs. Mock.

were subjected to 12 % SDS-PAGE, transferred to a PVDF membrane, and immunoblotted with anti-11β-HSD1 and anti-tubulin primary antibodies at 4 °C over night, followed by incubation with appropriate secondary antibodies, which were visualized with enhanced chemiluminescence (Pierce).

Statistics

Values are presented as the mean ± SD. The difference between groups was tested by Student's t test for two groups, or ANOVA for multiple groups. $p < 0.05$ was regarded as a significant difference.

Results

Lentivirus-mediated highly efficient delivery of 11β-HSD1 gene into MC3T3-L1 preosteoblasts

According to our previous study, 11β-HSD1 is expressed at a relatively low level in MC3T3-E1 cell line. In this study a four-plasmid based lentivirus system was used to overexpress 11β-HSD1 in MC3T3-E1 cell line. After infection of lentivirus and chemical selection by BSD, cells were collected to examine infection efficiency by real-time PCR and Western blot. The cells infected by negative control lentivirus were designated as Mock cells. As shown in Fig. 1A,

the relative expression level of 11β-HSD1 mRNA in the cells infected by the lentivirus expressing 11β-HSD1 was about 21.5-fold higher than that in Mock cells. Correspondingly, it was shown in Fig. 1B that the protein level of 11β-HSD1 in 11β-HSD1 lentivirus infected cells was about 2.5-fold over that in Mock cells, verifying efficient delivery of 11β-HSD1 gene into MC3T3-E1 preosteoblast.

11β-HSD1 overexpression inhibits osteoblastic differentiation of MC3T3-E1 cells

We first studied the effect of glucocorticoids in MC3T3-L1 differentiation. Under certain experimental conditions GCs promote osteoblast differentiation and matrix mineralization *in vitro* [18-20]. But more studies showed the inhibitory actions of GCs on the functional differentiation of osteoblasts as well as the stromal cells [21-25]. In our study, MC3T3-E1 cells were treated with dexamethasone (DEX) at different concentrations during osteogenic induction process. As demonstrated by Alizarin red staining in Fig. 2A, calcium deposition was obviously inhibited by DEX at a dose over 0.1 μM. We also performed dose response of active murine glucocorticoid, *i.e.* corticosterone as well as DEX on mineralization inhibition. In Fig. 2A, it was shown that mineralization was

Fig. 2 Dexamethasone (DEX) and corticosterone dose-dependently inhibits MC3T3-E1 osteogenesis.

MC3T3-E1 preosteoblasts were induced to osteogenesis at different concentration of DEX and corticosterone. Cells induced for 6 days were harvested for ALP activity and cells induced for 14 days were used for calcium deposit assay. (A) The mineral nodule was stained by 1 % alizarin red after fixed with paraformaldehyde. DEX and corticosterone dramatically inhibited formation of mineral nodule at doses higher than 0.1 μM and 1 μM, respectively. (B) Alkaline phosphatase (ALP) activities were measured as described in Materials and methods. DEX at doses higher than 0.01 μM significantly suppressed the ALP activity, consistently to results of mineralization. (C) DEX dose-dependently reduced mRNA expression levels of ALP, BSP, OPN and OCN. Relative expression level of each gene was normalized to 18S rRNA. Data were represented from three independent experiments with triplicates in each experiment. Error bar represents means ± SD. ** $p < 0.01$ vs. control.

dramatically inhibited by corticosterone at the dose of over 1 μM. As shown in Fig. 2B, the reduced ALP activity was observed when the cells were treated with DEX at the dose higher than 0.01 μM, consistently to the results of mineralization. For the effect on osteogenic marker gene expression, DEX dose-dependently reduced mRNA expression of mid-phase genes alkaline phosphatase (ALP) and bone sialoprotein (BSP), as well as the late-phase genes osteocalcin (OCN) and osteopontin (OPN) (Fig. 2C).

In contrast, the 11β-HSD1-overexpressing cells were induced to osteogenesis under the same condition with or without dehydrocorticosterone (DHC), the substrate of 11β-HSD1, which was converted from the inactive form to the active form of corticosterone. As first observed in Fig. 3, the differentiation ability toward osteogenesis of MC3T3-E1 cells was not influenced by lentivirus infection. There was no difference in mineralization between normal and Mock infected cells after 14-day osteogenic induction. It was also

Fig. 3 11 β -HSD1 overexpression inhibits mineralization in MC3T3-E1 cells.

MC3T3-E1 preosteoblasts were infected with lentivirus encoding 11 β -HSD1 gene and control lentivirus. After selected by BSD, cells were cultured to reach confluence and subjected to osteogenic induction with or without DHC substrate. Cells induced for 14 days were used for calcium deposit assay. The mineral nodule was stained by 1 % alizarin red after fixed with paraformaldehyde. Cells with 1 μ M DHC treatment were used for mineralization quantization. The red stain was released by ethylpyridium chloride extraction, and quantified by absorbance measurement at 550 nm. Cells with 11 β -HSD1 overexpression were significantly inhibited to form mineral nodule, compared to cells with Mock lentivirus transfection. Data were represented from three independent experiments with duplicates in each experiment. Error bar represents means $\pm$ SD. ** $p < 0.01$ vs. Mock.

noticed in Fig. 3 that there was no inhibition effect in Mock cells with different doses of DHC substrate. As expected, without the 11 β -HSD1 substrate, no suppression could be observed in 11 β -HSD1 overexpressed cells, either (data not shown). Similar to DEX treatment, DHC treatment inhibited calcium deposition in a dose-dependent manner at the dose higher than 1 μ M in 11 β -HSD1-overexpressing cells. Therefore, DHC dose

of 1 μ M was used in the following experiments. These results indicated that in the presence of DHC substrate, overexpressed 11 β -HSD1 could transfer inactive DHC to active corticosterone, mimicking an over-dosed GC and thereby inhibiting osteogenesis.

In addition, we observed no change in cell morphology during the osteogenic induction period. As shown in typical pictures in Supplementary Fig. 2, it was found the cells that failed to mineralize showed slower proliferation but with normal morphology in the end of osteogenesis.

11 β -HSD1 inhibitor BVT.2733 prevents osteoblastic dysfunction from overexpression of 11 β -HSD1 in MC3T3-E1 cells and primary murine osteoblasts

To determine if inhibition of 11 β -HSD1 activity could abolish the dysfunction of MC3T3-E1 cells overexpressing 11 β -HSD1 gene, a selective 11 β -HSD1 inhibitor BVT.2733 was used to treat cells that were induced for osteogenesis. After dose screening, a 100 μ M dose of BVT.2733 was chosen in the following experiments (Supplementary Fig. 3B). As shown in Fig. 4A, the mineral nodule staining and quantization indicated that BVT.2733 treatment made a significant abolishment of DHC inhibition effect. The ALP activity was significantly suppressed in DHC treated cells, while could be dramatically reversed by addition of BVT.2733 (Fig. 4B). Like DEX treatment of normal MC3T3-E1 cells, DHC treatment reduced mRNA expression of mid-phase genes ALP and BSP, as well as the late-phase genes OCN and OPN (Fig. 4C). However, when BVT.2733 was administered simultaneously, the expression levels of such osteogenic genes were all recovered to normal level. In addition, DEX induced inhibition could not be reversed by BVT.2733 treatment (data not shown). It could be easily explained by the notion that BVT.2733 inhibited the transformation of DHC to its active form, thus preventing the inhibition effect of 11 β -HSD1 overexpression. When DEX itself is in active form, BVT.2733 treatment could not abolish the inhibition effect of DEX.

We also reproduced the recovery effect of BVT.2733 in primary murine osteoblasts. In Fig. 5, the mineralization pattern is consistent with that in MC3T3 cell line. 11 β -HSD1 overexpression inhibited osteoblastic differentiation with the help of DHC substrate in primary murine osteoblasts, while BVT.2733 dramatically reversed this inhibition effect. This result further convinced our finding.

Fig. 4 BVT.2733 abrogates the inhibition effect of overexpression of 11β-HSD1 in MC3T3-E1 osteogenesis.

MC3T3-E1 preosteoblasts were infected with lentivirus encoding 11β-HSD1 gene and control lentivirus were subjected to osteogenic induction with DHC substrate and 11β-HSD1 inhibitor. Doses of DHC and BVT.2733 were 1 μM and 100 μM, respectively. (A) The mineral nodule staining and quantization were the same as Fig. 3. BVT.2733 treatment made a significant abolishment of DHC inhibition effect. (B) The ALP activity was significantly suppressed in DHC treated cells, while could be reversed by addition of BVT.2733. (C) DHC treatment reduced mRNA expression levels of ALP, BSP, OCN and OPN. However, when BVT.2733 was administered simultaneously, the expression levels of osteogenic genes were all recovered to normal values. Relative expression level of each gene was normalized to 18S rRNA. Data were represented from three independent experiments with duplicates in each experiment. Error bar represents means ±SD. ** $p < 0.01$ vs. Mock; ## $p < 0.01$ vs. 11β-HSD1 overexpressed cells without BVT.2733 treatment; NS means non significance.

GR antagonist RU486 rescues osteogenic activity in 11β-HSD1 overexpressed MC3T3-E1 cells

The above results indicated that the inhibition effect of osteogenic differentiation in 11β-HSD1-overexpressing MC3T3-E1 cells extremely mimicked the effect of GCs on osteogenesis. As the effects of GCs are primarily considered to be mediated by cytosolic glucocorticoid receptor (GR) activation, we further examined whether blocking the GR signal path-

way could alter suppression of osteogenic activity. RU486 was used as the GR blocker and its final concentration used in our study was 0.1 μM, as determined in Supplementary Fig. 3A when we first examined the effect of RU486 on the cells transfected with 11β-HSD1 lentivirus with addition of DHC. As shown in Fig. 6A, the inhibition effect of DHC on mineral nodule formation was significantly reversed by the treatment of RU486, which antagonized the GR signal. The sup-

Fig. 5 BVT.2733 recovered the ability of mineralization in primary murine osteoblasts inhibited by overexpression of 11 β -HSD1. Primary murine osteoblasts were prepared as described in the Method section and were infected with lentivirus encoding 11 β -HSD1 gene and control lentivirus exactly as MC3T3-E1 preosteoblasts. Cells were subjected to osteogenic induction with or without DHC substrate (1 μ M) or 11 β -HSD1 inhibitor BVT.2733 (100 μ M). Cells induced for 14 days were used for calcium deposit assay. The mineral nodus staining (left) and quantization (right) were the same as Fig. 3. BVT.2733 treatment showed a significant abolishment of DHC inhibition effect. Data of staining quantization were represented from three independent experiments with triplicates in each experiment. Error bar represents means $\pm$ SD. ** $p < 0.01$ vs. Mock; ## $p < 0.01$ vs. 11 β -HSD1 overexpressed cells without BVT.2733 treatment; NS means non significance.

pressed ALP activity in DHC-treated cells was also significantly abolished by addition of RU486 (Fig. 6B). With DHC present, the mRNA expression levels of osteogenic genes including ALP, BSP, OCN and OPN were all down-regulated in 11 β -HSD1 overexpressed cells, compared to gene levels in Mock cells (Fig. 6C). However, by RU486 involvement, the reduced mRNA levels were all recovered to normal levels, strongly confirming that the inhibition effect of 11 β -HSD1 overexpression was mediated through GR signal.

Discussion

Previous researches have shown that the primary causes of long-term GC-induced bone loss might be the direct inhibitory effects on osteoblasts. 11 β -HSD1 is the main regulator of GC activity in bone tissues. As 11 β -HSD1 expression and activity were both at a high level in aged animals, its precise role in the process of senile osteoporosis might be an object of concern. Till now, there has been only one report in which 11 β -HSD1 knockout mice were used to investigate the role of the intracellular activation of GCs on normal bone [15]. However, the investigation was performed in young mice and the results exhibited no significant bone mass changes in 11 β -HSD1 deficient mice compared with wild-type mice. Its role in senile ani-

mals maintains unknown. In this study, an 11 β -HSD1 overexpressed preosteoblast cell model was successfully established using lentivirus infection method. In this cell model, the osteogenic function was dramatically damaged, with reduced ALP activity, down-regulated osteogenic genes as well as mineralization level. Through BVT.2733 treatment, the excess 11 β -HSD1 could be effectively inhibited, thus reversed the destroyed osteogenesis under the condition of 11 β -HSD1 overexpression. This result gave us a hint the specific 11 β -HSD1 inhibitor might be an efficient agent to prevent senile osteoporosis.

We also confirmed that it was the GR signal which mediated the inhibition effect of 11 β -HSD1 overexpression. GC is well-known to be a very important immune regulator. 11 β -HSD1 is expressed in multiple tissues in human body, exerting different functions. If 11 β -HSD1 activity is inhibited without tissue specificity, GC level will be downregulated in the whole body, resulting in severe consequences, such as enhancement in immune response and reduction in endogenous anti-inflammatory effects [12, 26]. Therefore, when inhibiting 11 β -HSD1 activity, it is a huge challenge to improve bone metabolism without causing endogenous inflammatory response. In other words, it is necessary to look for a targeted bone-specific 11 β -HSD1 inhibitor. A suitable "warhead" guiding the drug molecule to

Fig. 6 DHC-induced osteogenic inhibition in MC3T3-E1 is abolished by RU486 treatment.

As described in Fig. 3, the infected MC3T3-E1 preosteoblasts were subjected to osteogenic induction with or without DHC substrate and GR blocker RU486. Doses of DHC and RU486 were 1 μ M and 0.1 μ M, respectively. (A) Assay of mineralization stain and quantization were the same as Fig. 3. The inhibition effects of DHC in 11 β -HSD1 overexpressed cells were significantly reversed by treatment of RU486, which antagonized the GR signal to form mineral nodus, compared to cells with Mock lentivirus transfection. (B) The suppressed ALP activity in DHC treated cells was also significantly abolished by addition of RU486. (C) The reduced mRNA expression of osteogenic genes including ALP, BSP, OCN and OPN could be recovered to the control levels by RU486 involvement. Relative expression level of each gene was normalized to 18S rRNA. Data were represented from three independent experiments with duplicates in each experiment. Error bar represents means $\pm$ SD. ** $p < 0.01$ vs. Mock; ## $p < 0.01$ vs. 11 β -HSD1 overexpressed cells without RU486 treatment; NS means non significance.

bone tissue is the key to solve this problem.

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Competing Interests

The authors have declared that no competing interests exist.

Supplementary Fig. 1 Plasmid pGRE-luc was transfected to 3T3-L1 fibroblasts, in which 11β -HSD1 activity is very high.

Luciferase activities were assayed 24 h after transfection. In the presence of 11β -HSD1 substrate DHC, inactive glucocorticoid DHC was converted to the active form by the endogenous 11β -HSD1 activity, and therefore inducing up-regulated GRE-Luc activity as well as the active corticosterone. However, addition of BVT.2733 reversed the up-regulated luciferase activity induced by DHC. By contrast, the luciferase activity induced by corticosterone was not affected by BVT.2733 treatment. Dose of BVT.2733 was 100 μ M. Data were represented from three independent experiments with triplicates in each experiment. Error bar represents means $\pm$ SD. ** $p < 0.01$ compared to control. ## $p < 0.01$ compared to BVT.2733 untreated.

Supplementary Fig. 2 Typical photos of MC3T3 cells taken in the end of osteogenic induction.

(A) Cells infected with mock lentivirus showed wonderful calcium deposition. (B) Mineralization in cells infected with 11β -HSD1 overexpressing lentivirus and treated with addition of 1 μ M DHC substrate was dramatically inhibited. (C) Cells treated with 0.1 μ M DEX during osteogenic induction were totally inhibited to osteogenesis. During the period, cells were observed with slower proliferation but without obvious death.

Supplementary Fig. 3 DHC-induced osteogenic inhibition in 11b-HSD1 overexpressed MC3T3-E1 is abolished by RU486 treatment or BVT.2733 treatment.

As described in Fig. 3, the infected MC3T3-E1 preosteoblasts were subjected to osteogenic induction with 1 μM DHC substrate and GR blocker RU486 (A) or BVT.2733 (B) in different doses. Cells induced for 14 days were used for mineralization staining. (A) The inhibition effect was significantly reversed by treatment of 0.1 μM RU486. However, high doses of RU486 totally blocked the GR signal and caused failure of mineralization. (B) 100 μM of BVT.2733 recovered inhibited osteogenesis most efficiently. High doses seemed to show cell toxicity and caused failure of mineralization. Therefore, 100 μM BVT.2733 was used in the following experiments.

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