A novel 11β-HSD1 inhibitor improves diabesity and osteoblast differentiation

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Abstract
Selective inhibitors of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) have considerable potential as treatment for osteoporosis as well as metabolic syndrome including type 2 diabetes mellitus. Here, we investigated the anti-diabetic, anti-adipogenic, and anti-osteoporotic activity of KR-67500, as a novel selective 11β-HSD1 inhibitor. Cellular 11β-HSD1 activity was tested based on a homogeneous time-resolved fluorescence method. Oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) levels were measured in diet-induced obese (DIO)-C57BL/6 mice administered KR-67500 (50 mg/kg per day, p.o.) for 28 days and, additionally, its anti-diabetic effect was evaluated by OGTT and ITT. The in vitro anti-adipogenic effect of KR-67500 was determined by Oil Red O Staining. The in vitro anti-osteoporotic activity of KR-67500 was evaluated using bone morphogenetic protein 2 (BMP2)-induced osteoblast differentiation and receptor activator of nuclear factor-κB ligand (RANKL)-induced osteoclast differentiation model systems. KR-67500 improved the in vivo glucose tolerance and insulin sensitivity in DIO-C57BL/6 mice. KR-67500 suppressed cortisone-induced differentiation of 3T3-L1 cells into adipocytes. KR-67500 enhanced BMP2-induced osteoblastogenesis in C2C12 cells and inhibited RANKL-induced osteoclastogenesis in mouse bone marrow-derived macrophages. KR-67500, a new selective 11β-HSD1 inhibitor, may provide a new therapeutic window in the prevention and/or treatment of type 2 diabetes, obesity, and/or osteoporosis.

Introduction
11β-hydroxysteroid dehydrogenase enzyme (11β-HSD) regulates the level of glucocorticoid by converting cortisol (corticosterone in rodents), but its dysfunction or an excess of glucocorticoid causes several metabolic disorders (Stewart & Krozowski 1999, Wang 2005). The relationship
between 11β-HSD1 and type 2 diabetes mellitus has been demonstrated in mouse genetic models (Masuzaki et al. 2001, Morton et al. 2004). Adipose tissue in 11β-HSD1-overexpressed mice showed metabolic syndrome-like phenotypes such as central obesity, glucose tolerance, and insulin resistance (Masuzaki et al. 2001, Paterson et al. 2004). By contrast, 11β-HSD1-deficient mice showed a reduction in high-fat diet-induced obesity with improved insulin sensitivity and lipid profiles (Kotelevtsev et al. 1997, Morton et al. 2001, 2004).

Furthermore, glucocorticoids are essential for terminal adipogenesis (Hauner et al. 1987) and limit cell proliferation in preadipocytes (Tomlinson et al. 2006). Mature adipocytes express late differentiation genes involved in lipid metabolism and lipid transport, and many of these genes are regulated by glucocorticoids (Wu et al. 2000, Park et al. 2005, 2011, Rosen & MacDougald 2006).

Additionally, several studies showed that any condition with excess glucocorticoid leads to decreased bone mineral density and increased risk of fracture (Dalle Carbonare et al. 2001, Canalis et al. 2007). Glucocorticoid promotes the expression of receptor activator of nuclear factor-κB ligand (RANKL) and decreases osteoprotegerin expression in osteoblasts, thus stimulating osteoclastic bone resorption (Hofbauer et al. 1999). Additionally, glucocorticoid suppresses intestinal absorption of calcium and gonadal hormones (Klein et al. 1977).

In a previous study, we developed a series of cyclic sulfamide derivatives with an adamantyl group as 11β-HSD1 inhibitors (Kim et al. 2012). Among cyclic sulfamide derivatives with an adamantyl group, trans-4-(2-(4-methyl-1,1-dioxido-6-(2,4,6-trichlorophenyl)-1,2,6-thiadiazinan-2-yl)acetamido) adamantane-1-carboxamide (KR-67500) showed potent in vitro activity against human and mouse 11β-HSD1 with IC_{50} values of 1 and 2 nM and exhibited selectivity toward 11β-HSD2 (Kim et al. 2012). Furthermore, KR-67500 showed good metabolic stability during incubation of human and mouse liver microsome, weak inhibition of cardiototoxicity, no cytochrome P450 inhibition (1A2, 2C9, 2C19, 2D6, and 3A4), and a LD_{50} value of over 1000 mg/kg in ICR mice. Additionally, following oral administration of 10 mg/kg in rats, KR-67500 exhibited systemic clearance of 0.7 l/h per kg, with terminal half-life of 3.8 h. The absolute oral bioavailability of KR-67500 was 68.8% in rats. Similarly, following oral administration of 10 mg/kg in cynomologous monkey, KR-67500 exhibited the maximum concentration of 6.4 μg/ml, with terminal half-life of 3.4 h (Kim et al. 2012).

Here, we have explored whether KR-67500 can improve glucose tolerance and insulin sensitivity in diet-induced obese (DIO)-C57BL/6 mice. Additionally, we accessed the effect of KR-67500 on the cortisone-induced adipogenesis in 3T3-L1 cells, the bone morphogenetic protein 2 (BMP2)-induced osteoblastogenesis in C2C12 cells, and the RANKL-induced osteoclast differentiation in mouse bone marrow-derived macrophages.

**Materials and methods**

**Drugs**

KR-67500 compounds were synthesized in the Korea Research Institute of Chemical Technology (Daejon, Korea). Carbenoxolone (CBX) purchased from Amifenecom, Inc. (Petersburg, VA, USA) was used as a reference agent in this study. Cortisone and NADPH were from Sigma–Aldrich.

**Animals and drug administration**

All animals were maintained in a room illuminated daily from 0700 to 1900 h (12 h light:12 h darkness cycle), with controlled temperature (23 ± 1 °C) and ventilation (10–12 times per hour), and humidity was maintained at 55 ± 5%. Mice were caged individually and allowed free access to food and tap water in accordance with the NIH Guide for the Care and Use of Laboratory Animals (http://oacu.od.nih.gov/ac_chf/guide3.htm). The C57BL/6 mice used in the experiments were purchased from the Orient Bio (Seongnam, Korea). For DIO induction, male C57BL/6 mice were fed with a high-fat diet containing 40% (w/w) of total fat (60% of total energy from fat, purchased from Diet Research, Inc. (New Brunswick, NJ, USA)) for 12 weeks, starting at 4 weeks of age. The effects of the compounds were tested in DIO-C57BL/6 mice, 16–20 weeks in age. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Korea Research Institute of Chemical Technology, the Ministry of Knowledge Economy, Korea (Permit Number: 2009-7A-09-03).

Animals were weighted regularly to allow accurate dosing with vehicle (0.5% carboxymethylcellulose sodium (CMC) in H_{2}O) or KR-67500. KR-67500 was mixed with the vehicles, ground in a mortar, and sonicated to improve the solubility of the compound. Male DIO-C57BL/6 mice were orally gavaged for a week to acclimatize to the vehicle. KR-67500 (50 mg/kg BW) was orally administered twice daily at 0900 and 1700 h for 4 weeks. After 4 weeks of treatment with the drug, the anti-diabetic effect of KR-67500 was measured using the oral glucose tolerance test (OGTT) and the insulin tolerance test (ITT).
OGTT and ITT

We examined the OGTT and ITT as described previously (Park et al. 2012). The plasma concentration of glucose was measured by colorimetric assay using an automatic biochemical analyzer, the Selectra 2 (Vital Scientific N.V., Spankeren/Dieren, Netherlands). The plasma concentrations of insulin were measured using the insulin assay kit of Shibayagi Co. (Gunma, Japan). HOMA-IR index was calculated from the fasting glucose (mg/dl) and fasting insulin concentration (µIU/ml) by the equation described elsewhere (fasting insulin x fasting glucose)/405 (Tachibe et al. 2009).

Cell culture, differentiation, and staining

Murine 3T3-L1 (CL-173; ATCC, Manassass, VA, USA) preadipocytes were cultured and differentiated into adipocytes as described previously (Park et al. 2012).

Murine bi-potential mesenchymal precursor C2C12 cells (ATCC CRL-1772) were cultured and differentiated into osteoblasts as described in a previous study (Kim & Kim 2010). Briefly, cells were seeded at a density of 4 x 10^3 or 1.5 x 10^5 cells/well in 96-well plate and six-well culture plates respectively. After 1-day of incubation, the osteoblastogenesis was triggered by the medium containing 5% FBS and rhBMP-2 (50 ng/ml; R&D Systems, Minneapolis, MN, USA). An Alkaline Phosphatase (ALP) Kit (Sigma–Aldrich) was used to visualize cellular ALP expression.

BMMs-based osteoclast differentiation and TRAP activity assay were carried out as described in a previous study (Choi et al. 2013).

Lipid accumulation assay

The cellular lipid content was assessed by Oil Red O staining (Sigma–Aldrich). After 7 days, cells were washed and fixed in 1% paraformaldehyde for 1 h, stained with Oil Red O working solution, and incubated for another 4 h at 37 °C. After being washed three times with PBS, the cells were photographed using a light microscope (Olympus, Tokyo, Japan).

Real-time quantitative PCR analysis

We examined the gene expressions as described previously (Park et al. 2011). Expression studies were carried out using gene-specific primers for mouse peroxisome proliferator-activated receptor γ (Pparγ2), glycerol-3-phosphate dehydrogenase (Gpd1), fatty acid binding protein 4 (Fabp4), glucose transporter 4 (Slc2a4), Hsd11b1, Alppl2, Bmp4, Acp5, Fos, nuclear factor of activated T cells c1 (Nfatc1), Ctsk, and dendrite cell-specific transmembrane protein (Dcstamp). All primers were designed using primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/) and their sequences from 5’ to 3’ are shown in Table 1. The relative abundance of mRNA was calculated after normalization to glyceraldehyde-3-phosphate dehydrogenase (Gapdh).

In vitro 11β-HSD1 enzyme activity assay

We examined the cellular 11β-HSD1 enzyme activity as described previously (Cho et al. 2009). Differentiated C2C12 cells for 3 days in the presence of BMP2 (50 ng/ml) and 3T3-L1 adipocytes were seeded at 2 x 10^3 cells/well or 5 x 10^5 cells/well onto 96- or 24-well plates and incubated in a medium containing 160 nM cortisone in the presence or absence of compounds 24 h (3T3-L1 cells) and 48 h (C2C12 cells). Small aliquots (10 µl) of the reaction mixtures were removed and subjected to homogeneous time-resolved fluorescence cortisol assay in accordance with the manufacturer’s instructions (Nihon Schering, Tokyo, Japan). The IC_{50} values of the compounds were determined from concentration-dependent inhibition curves by GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). CBX was used as the reference compounds.

Ex vivo 11β-HSD1 enzyme activity assay

We examined the ex vivo 11β-HSD1 enzyme activity as described previously (Park et al. 2012). Male C57BL/6 mice, 12 weeks old, were orally gavaged with vehicle (0.5% CMC/0.1% Tween 80 in H2O) or KR-67500 at 0.5, 1, 5, and 10 mg/kg and killed 2 or 6 h (10 mg/kg) after dose (n = 4 per group).

Table 1 Primer sequences for various mouse genes for real-time PCR. Primers were designed using Primer3 software (http://bioinfo.ut.ee/primer3-0.4.0/)

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Statistical analysis

All data are expressed as mean ± S.E.M. Statistical significance was determined by one-way ANOVA followed by a Turkey’s multiple-comparison test. *P*<0.05 was considered to be statistically significant.

Results

Effect of KR-67500 on glucose tolerance and insulin sensitivity in DIO-C57BL/6 mice

We evaluated whether the inhibitory activity of KR-67500 on 11β-HSD1 ameliorated glucose tolerance and insulin resistance in DIO-C57BL/6 mice after treatment with KR-67500 (50 mg/kg per day, orally for 28 days). Oral treatment with KR-67500 resulted in increased glucose clearance and plasma glucose levels, as determined by the area under the glucose concentration curve (AUC), were significantly reduced by 10.6% compared with the vehicle by administration of KR-67500 (Fig. 1A). Using the same mouse model, we measured the insulin sensitivity by glucose intolerance test (ITT) (Fig. 1B). The mean AUC of the KR-67500-treated mice was also significantly (*P*<0.05) lower than that of the vehicle-treated mice. Furthermore, in these mice, the plasma fasting insulin concentration and HOMA-IR index of the KR-67500-treated mice was lower than that of the vehicle-treated DIO-C57BL/6 mice (Fig. 1C and D). In this manner, treatment of KR-67500 improved plasma lipid profiles such as triglyceride (TG) and HDL- and LDL-cholesterol in the vehicle-treated DIO-C57BL/6 mice after 28 days (Table 2).

Additionally, KR-67500 inhibited the 11β-HSD1 enzyme activity in liver, inguinal fat, and reproductive fat of DIO-C57BL/6 mice (Fig. 1E).

Effect of KR-67500 on cortisone-induced adipogenesis in 3T3-L1 cells

Adipocytes with 1 µM cortisone showed increased lipid accumulation when assessed after 7 days (data not shown). This effect was removed by co-incubation with KR-67500 in a concentration-dependent manner (Fig. 2A) without apparent cytotoxicity (data not shown).

Furthermore, we examined whether the mRNA expression of adipogenesis-specific genes is inhibited by KR-67500. On day 7, real-time PCR was performed to analyze the mRNA levels of key transcription factors in adipogenesis such as *Pparγ*, *Gpd1*, *Fahp4*, and *Sreca4*. As shown in Fig. 2B, KR-67500 significantly downregulated the overexpression of all these genes by 1 µM cortisone in a concentration-dependent manner.

In Fig. 3A, KR-67500 inhibited the 11β-HSD1 activity of 3T3-L1 adipocytes in a concentration-dependent manner, and the IC_{50} value of KR-67500 was 4.3 nM. The IC_{50} value of CBX was 170 nM. Treatment of KR-67500 also inhibited the induction of 11β-HSD1 expression by cortisone in 3T3-L1 adipocytes. In this manner, KR-67500 was administered to lean C57BL/6 mice in a single 0.5, 1, 5, and 10 mg/kg oral dose, and after 2 h, the inhibition of 11β-HSD1 enzyme activity of inguinal fat was measured *ex vivo* by incubating fat in media containing cortisone and NADPH. KR-67500 inhibited in a concentration-dependent manner, with >50% inhibition at 0.5 mg/kg (Fig. 3B). Furthermore, treatment of 10 mg/kg KR-67500 also significantly inhibited the 11β-HSD1 enzyme activity at 2 and 6 h in inguinal and reproductive fats (Fig. 3C).

Effect of KR-67500 on osteoblast differentiation in C2C12 cells

In C2C12 cells, KR-67500 dose-dependently enhanced the BMP2-induced expression of ALP, a biomarker of osteoblastogenesis (Fig. 4A) without apparent cytotoxicity (data not shown). Osteogenic activity of KR-67500 was stronger than that of CBX at 10 µM. Neither KR-67500 nor CBX exhibited the osteogenic activity in the absence of BMP2. The osteogenic activity of KR-67500 was confirmed by real-time PCR analysis (Fig. 4B); the BMP2-induced mRNA expression levels of *Alpl* and *Bmp4* were significantly enhanced by the addition of KR-67500.

Furthermore, on differentiation day 3, the cellular oxo-reductase activity of 11β-HSD1 in C2C12 cells was significantly inhibited by KR-67500 with >50% inhibition at 0.001 µM (Fig. 5A). An inhibitory effect of CBX on the activity of 11β-HSD1 was also observed, but it was weaker than that of KR-67500. The mRNA expression of 11β-HSD1 was significantly inhibited by BMP2, and its inhibition was stronger in cells treated with BMP-2 and KR-67500 (Fig. 5B). Thus, these results suggested that KR-67500 could enhance the BMP2-induced osteoblast differentiation with the inhibition of 11β-HSD1 enzyme expression and activity.

Effect of KR-67500 on osteoclast differentiation in BMMs

In the BMM-based osteoclastogenesis model, the effect of KR-67500 on the osteoclast differentiation was evaluated by staining TRAP and measuring its activity. TRAP is a
Figure 1
Anti-diabetic effect of KR-67500 on glucose tolerance and insulin sensitivity in DIO-C57BL/6 mice. DIO-C57BL/6 mice were administered with vehicle and KR-67500 (50 mg/kg BW) daily by oral gavage for 28 days. (A) The oral glucose tolerance test. (B) The insulin tolerance test. (closed circle) DIO-C57BL/6 – vehicle, (closed square) DIO-C57BL/6 – KR-67500. (C) Plasma fasting insulin concentration. (D) HOMA-IR index analysis. (E) Ex vivo 11β-HSD1 enzyme activity in liver, inguinal fat, and reproductive fat of KR-67500 treated DIO-C57BL/6 mice. The ex vivo assay was carried out at 16 h after the last administration in the in vivo DIO-C57BL/6 mice study. Results are expressed as mean ± S.E.M. for n=6–9 mice per group. *P<0.05, **P<0.01, ***P<0.001 vs DIO-C57BL/6 vehicle group.
Table 2  Metabolic parameters of mice with KR-67500. Values are mean ± S.E.M.; n = 6–8/group

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<tr>
<td>Cholesterol (mg/dl)</td>
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<td>TG (mg/dl)</td>
<td>86.2 ± 9.4</td>
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<td>HDL (mg/dl)</td>
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<td>LDL (mg/dl)</td>
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<td>65.6 ± 3.4*</td>
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TG, triglyceride; FFA, free fatty acid. *P < 0.05 vs DIO-vehicle group.

well-known biomarker of osteoclastogenesis. As shown in Fig. 6A, BMMs differentiated into TRAP-positive multinucleated osteoclasts in response to M-CSF and RANKL, but KR-67500 dose-dependently inhibited the formation of TRAP-positive multinucleated osteoclasts without apparent cytotoxicity (data not shown). CBX did not exhibit any anti-osteoclastogenic action.

TRAP activity assay and the real-time PCR analysis revealed that KR-67500 dose-dependently inhibited the TRAP activity (Fig. 6B) and significantly attenuated the RANKL-induced mRNA expression of genes related to fusion (Fos, Nfatc1, and Dcstamp) and function (Ctsk) of osteoclasts (Fig. 6C). However, the mRNA expression of 11β-HSD1-related genes (Hsd11b1, gr, and H6pd) were not changed by KR-67500 in the process of osteoclastogenesis (data not shown).

Discussion
Numerous rodent studies have demonstrated the potential use of 11β-HSD1 inhibitors as treatment for the components of metabolic syndrome, and limited clinical data on humans have shown 11β-HSD1 inhibition to improve glucose levels, insulin sensitivity, and lipid profiles.

Figure 2
Anti-adipogenic effect of KR-67500 on lipid accumulation (A) and adipogenic markers expression (B) in cortisone-treated 3T3-L1 cells. Preadipocytes (control) were induced to differentiate with 20 µg/ml insulin, 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone, 1 µM cortisone, and KR-67500 (10 and 20 µM), or CBX (20 µM) for 3 days, which was then replaced with medium containing 1 µM cortisone, 20 µg/ml insulin, and compounds for 2 days, and the cells were then cultured for 1 day in culture medium. Values are mean ± S.E.M. of data from two separate experiments with three replicates. ### P < 0.001 vs control group; *P < 0.05, **P < 0.01, ###P < 0.001 vs vehicle group. A full colour version of this figure is available via http://dx.doi.org/10.1530/JME-13-0177.
In this study, we have explored whether a novel 11β-HSD1 inhibitor, KR-67500, can improve glucose tolerance and insulin sensitivity in DIO-C57BL/6 mice and additionally prevent the cortisone-induced adipogenesis, the BMP2-induced C2C12 osteogenesis, and the RANKL-induced osteoclast differentiation via the inhibition of 11β-HSD1 enzyme activity.

The relationships between 11β-HSD1 and type 2 diabetes have been demonstrated in mouse genetic models. The 11β-HSD1-overexpressing transgenic mice in adipose tissues showed metabolic syndrome-like phenotypes such as central obesity, glucose intolerance, and insulin resistance (Masuzaki et al. 2001), but those tissues from Hsd11b1 knock-out mice showed a reduction in high-fat diet-induced obesity with improved insulin sensitivity and lipid profiles (Morton et al. 2004). Furthermore, Rosenstock et al. (2010) published the results of a 12-week efficacy study evaluating INCB13739 (Incyte Corporation, Wilmington, DE, USA), a potent selective 11β-HSD1 inhibitor, in patients with type 2 diabetes mellitus who were failing metformin monotherapy. In this study, oral treatment with KR-67500, a novel selective 11β-HSD1 inhibitor,
exhibited significantly improved glucose tolerance and insulin sensitivity, as determined through several assays including an OGTT, ITT, plasma fasting insulin concentration assay, and HOMA-IR index analysis in DIO-C57BL/6 mice. In this manner, treatment of KR-67500 improved plasma lipid profiles such as TG and HDL- and LDL-cholesterol in the vehicle-treated DIO-C57BL/6 mice after 28 days. Additionally, KR-67500 significantly inhibited 11β-HSD1 activity in the liver and fat of DIO-C57BL/6 mice. Considering these results, it is suggested that the improvement of glucose tolerance and insulin sensitivity by KR-67500 in diabetic mice can be ascribed to the inhibition of 11β-HSD1 enzyme activity in the liver and fat.

Recent investigations showed that 11β-HSD1 inhibitors prevented adipogenesis with beneficial application to the treatment of obesity in diabetic patients (Bujalska et al. 2008). Mature adipocytes express late differentiation genes involved in lipid metabolism and lipid transport, including Gpd1 and Fabp4 (Hotamisligil et al. 1996), and many of these genes are regulated by glucocorticoids (Wu et al. 1996, Rosen & MacDougald 2006). PPARG was shown to play an important role in adipocyte differentiation, glucose homeostasis, and insulin signaling (Kersten et al. 2000). Moreover, glucose transporter, type 4 (GLUT4) is selectively expressed in insulin-sensitive tissues such as muscle and adipose cells (Bryant et al. 2002) and impairment of glucose uptake by adipose and muscle tissues in obesity is associated with the reduction of cellular GLUT4 content (Sato et al. 1997), while a loss of body weight from diet therapy greatly improves abnormal glucose homeostasis (Wing et al. 1994). In our results, preadipocytes with 1 μM cortisone showed increased lipid accumulation when assessed after 7 days and these effects were eliminated by co-incubation with KR-67500. In line with this, the mRNA expression of adipogenesis-specific genes was inhibited by KR-67500; the mRNA levels of Pparg, Gpd1, Fabp4, and Slc2a4 was increased by cortisone; and these inductions were inhibited by KR-67500. Moreover, KR-67500 inhibited Hsd11b1 mRNA expression and enzyme activity in adipocytes. Furthermore, in ex vivo 11β-HSD1 activity assay of inguinal fats of C57BL/6 mice, KR-67500 inhibited in a concentration-dependent manner, with >50% inhibition at 0.5 mg/kg. Treatment of 10 mg/kg KR-67500 also significantly inhibited the 11β-HSD1 enzyme activity at 2 and 6 h in inguinal and reproductive tissue.
Inhibitory effect of KR-67500 on the activity (A) and the expression of 11β-HSD1 in differentiated C2C12 cells (B). (A) Mouse-differentiated C2C12 cells were seeded at 2×10^4 cells/well onto 96-well plates and incubated in a medium containing 160 nM cortisone in the presence or absence of compounds for 48 h at 37 °C. Results are expressed as mean ± S.E.M. of triplicate experiments. ***P<0.001 vs cortisone-treated group. (B) Effect of KR-67500 on mRNA 11β-HSD1 expression in BMP2-treated C2C12 cells. The mRNA 11β-HSD1 expression was determined by real-time PCR. Values are mean ± S.E.M. of data from two separate experiments with three replicates. ###P<0.001 vs control group; *P<0.05 vs BMP2-treated group.

Figure 5

In summary, KR-67500, a novel 11β-HSD1 inhibitor, exhibits the anti-diabetic and anti-adipogenic activities. Also, KR-67500 enhances the osteoblastogenesis and inhibits the osteoclastogenesis. A novel selective 11β-HSD1 inhibitor may provide a new therapeutic window in the prevention and treatment of type 2 diabetes patients with obesity and osteoporosis.
Figure 6
Anti-osteoclastogenic activity of KR-67500. BMM cells were cultured for 4 days in the presence of RANKL (5 ng/ml) and M-CSF (30 ng/ml) with KR-67500 or CBX. Multinucleated osteoclasts were visualized to red-colored giant cells by TRAP staining (A) and their activity was also measured (B). (C) Effect of KR-67500 on osteoclastogenesis-related gene expressions. After treating the vehicle (DMSO) or KR-67500 (5 μM) for 1 h, BMMs were treated with RANKL (5 ng/ml) for the indicated number of days and then mRNA expression levels were analyzed by real-time PCR. *P < 0.05, **P < 0.01, ***P < 0.001 vs control group. A full colour version of this figure is available via http://dx.doi.org/10.1530/JME-13-0177.
**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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