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Regulation of bone mass, bone loss and osteoclast activity by cannabinoid receptors

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Accelerated osteoclastic bone resorption has a central role in the pathogenesis of osteoporosis and other bone diseases. Identifying the molecular pathways that regulate osteoclast activity provides a key to understanding the causes of these diseases and to the development of new treatments. Here we show that mice with inactivation of cannabinoid type 1 (CB_1) receptors have increased bone mass and are protected from ovariectomy-induced bone loss. Pharmacological antagonists of CB_1 and CB_2 receptors prevented ovariectomy-induced bone loss *in vivo* and caused osteoclast inhibition *in vitro* by promoting osteoclast apoptosis and inhibiting production of several osteoclast survival factors. These studies show that the CB_1 receptor has a role in the regulation of bone mass and ovariectomy-induced bone loss and that CB_1- and CB_2-selective cannabinoid receptor antagonists are a new class of osteoclast inhibitors that may be of value in the treatment of osteoporosis and other bone diseases.

Osteoclasts are cells derived from the monocyte-macrophage lineage that have an important role in modeling bone during skeletal growth and in remodeling bone during adult life[1]. Increased osteoclast activity or uncoupling of osteoclastic bone resorption from bone formation results in focal or generalized bone loss and is a characteristic feature of bone diseases such as osteoporosis, Paget disease of bone and cancer-associated bone disease[2]. The importance of osteoclastic bone resorption in the pathogenesis of these diseases is reflected by the fact that the most successful drug treatments for bone disease work by inhibiting bone resorption[3]. Osteoclastic bone resorption is regulated by a complex interplay between circulating calciotropic hormones like parathyroid hormone, calcitriol and sex hormones, and local regulators of bone cell activity like receptor activator of nuclear factor κB ligand (RANKL), macrophage colony-stimulating factor (M-CSF) and osteoprotegerin[4]. Recent work has shown that neuroendocrine pathways and neurotransmitters also have a key role in the regulation of bone remodeling[5–9]. In view of this, we investigated the role of the endocannabinoid system in the regulation of bone mass and bone turnover by studying the skeletal phenotype in mice with targeted inactivation of CB_1 receptors (CB_1 knockout mice) and by studying the effects of cannabinoid receptor ligands on bone cell function *in vitro* and ovariectomy-induced bone loss *in vivo*.

RESULTS

Skeletal phenotype of CB_1 knockout mice

We found that CB_1 knockout mice had significantly increased bone mineral density (BMD) when compared with wild-type littermates. For example, the levels of BMD assessed by dual X-ray absorptiometry at the femur were 18% higher ($P < 0.001$) in CB_1 knockout animals than wild-type and values at the spine were 10% higher ($P < 0.02$; **Fig. 1a**). Further evaluation using peripheral quantitative computed tomography showed that trabecular BMD values at the tibial metaphysis in CB_1 knockout mice were 16% higher than in wild-type ($P < 0.001$; **Fig. 1b**) and this difference was clearly evident on low-power photomicrographs at this site (**Fig. 1c**). Bone histomorphometry showed that CB_1 knockout mice had significantly increased trabecular bone volume at the tibial metaphysis compared with wild-type mice ($P < 0.001$), consistent with the BMD measurements, but no difference was observed between CB_1 knockout mice and wild-type littermates in terms of osteoclast numbers, eroded surfaces or osteoblast numbers (**Supplementary Table 1** online). This indicates that absence of the CB_1 receptor regulates BMD but does not influence bone turnover appreciably in normal adult mice. To determine whether the CB_1 receptor has a role in the regulation of bone loss, we studied the effects of ovariectomy in CB_1 knockout and wild-type mice. We found that CB_1 knockout mice were completely resistant to trabecular bone loss induced by ovariectomy when compared with wild-type; analysis by peripheral quantitative computed tomography showed no significant change in total BMD of the tibial metaphysis in CB_1 knockout mice after ovariectomy, compared with a 12% reduction in wild-type ($P < 0.01$; **Fig. 2a**). Analysis by microcomputed tomography showed a 40% reduction in trabecular bone volume after ovariectomy in wild-type mice ($P < 0.001$; **Fig. 2b**), a 12% reduction in trabecular thickness ($P < 0.05$; **Fig. 2c**) and a 30% reduction in trabecular number ($P < 0.01$; **Fig. 2d**), whereas none of these variables changed significantly in CB_1 knockout mice. Uterine weight

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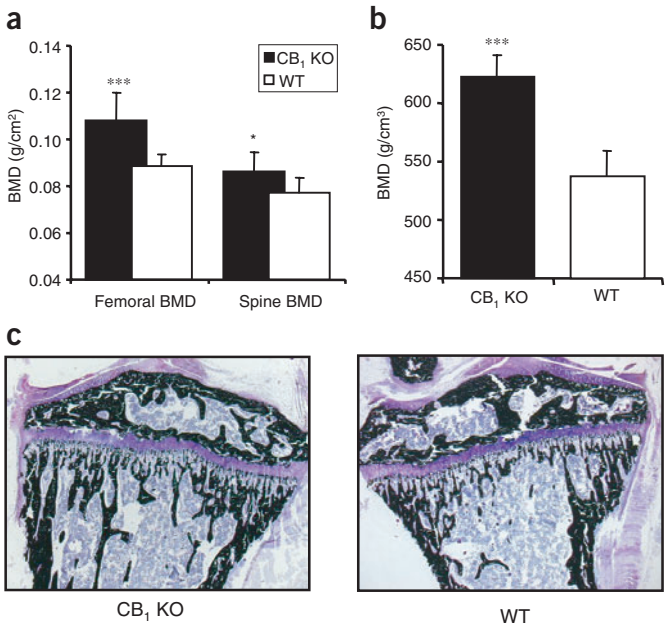

Figure 1 CB_1 knockout mice have increased bone mass. (**a**) BMD at the spine and femur assessed by dual X-ray absorptiometry in CB_1 knockout (KO) mice and wild-type (WT) littermates. (**b**) Trabecular BMD at the tibial metaphysis assessed by peripheral quantitative computed tomography in CB_1 knockout mice and wild-type littermates. (**c**) Representative photomicrograph of the proximal tibia from and CB_1 knockout mice (left) and wild-type mice (right). Data are presented as mean + s.e.m. $^{***}P < 0.001$, $^{*}P < 0.02$ comparing CB_1 knockout and wild-type mice.

(mean ± s.d.) fell to a similar degree after ovariectomy in wild-type mice (0.38 ± 0.03 versus 0.08 ± 0.01 g; $P < 0.01$) and CB_1 knockout mice (0.43 ± 0.02 versus 0.09 ± 0.01 g; $P < 0.01$). These data indicate that the CB_1 receptor has an essential role in regulating the bone loss that results from estrogen deficiency, but that the gonadal response to ovariectomy is unaffected by CB_1 deficiency.

Effects of cannabinoid receptor ligands on osteoclast function

To explore the mechanisms by which the CB_1 pathway regulates bone mass and bone loss, we studied the effects of various cannabinoid receptor agonists and antagonists on bone cell function *in vitro* using primary mouse osteoblast cultures and RANKL-generated mouse osteoclast cultures. None of the cannabinoid ligands that we tested significantly affected osteoblast growth or viability at concentrations of up to 20 µM (data not shown), but we did observe significant effects on osteoclast activity when we used ligand concentrations in the nanomolar range. The CB_1-selective antagonist AM251 (ref. 10) and the CB_2-selective antagonists SR144528 and AM630 (ref. 10) significantly inhibited osteoclast formation in RANKL- and M-CSF–stimulated mouse bone marrow cultures in a concentration-dependent manner with 50% inhibition at 700 nM for AM251, 850 nM for SR144528 and 100 nM for AM630 ($P < 0.05$; **Fig. 3a**). Conversely, the endogenous cannabinoid receptor agonist anandamide and the nonselective synthetic agonist CP55940 stimulated osteoclast formation in a concentration-dependent manner between 100 nM and 5 µM (**Fig. 3a**). Representative photomicrographs from cultures treated with vehicle, anandamide, AM251 and SR144528 are shown in **Fig. 3b**. Moreover, anandamide reversed the inhibitory effect of AM251 on osteoclast formation (**Fig. 3c**), consistent with a receptor-mediated effect.

Functional role of CB_1 and CB_2 receptors on osteoclasts

Both the CB_1 and CB_2 receptor subtypes were expressed on mouse osteoclasts as assessed by immunohistochemical staining, western blotting and RT-PCR (**Supplementary Fig. 1** online), indicating that CB_1, CB_2 or both receptors could mediate the effects of cannabinoid ligands on osteoclast activity. To clarify the relative role of the CB_1 and CB_2 receptors as targets for the regulation of osteoclast activity, we compared the effects of AM251 and AM630 on RANKL-induced osteoclast formation in cultures generated from CB_1 knockout mice and wild-type littermates. Cultures prepared from CB_1 knockout mice were resistant to the inhibitory effects of AM251 on osteoclast formation when compared with wild-type cultures (**Fig. 4a**). The CB_2-selective antagonist AM630 inhibited osteoclast formation to a similar extent in cultures prepared from CB_1 knockout and wild-type mice and was an order of magnitude more potent than AM251 (**Fig. 4b**). Taken together, these data indicate that the CB_1 receptor contributes in part to the osteoclast inhibition that occurs *in vitro* when these cultures are exposed to cannabinoid antagonists, but that osteoclast inhibition can also occur through antagonism of CB_2 receptors.

Effects of cannabinoid antagonists on bone loss

As CB_1 knockout mice were resistant to ovariectomy-induced bone loss, we wanted to determine whether pharmacological blockade of cannabinoid receptors could prevent the bone loss that occurs as the result of ovariectomy. We studied the effects of AM251 in doses of 0.3–3.0 mg/kg/d on ovariectomy-induced bone loss in wild-type mice and SR144528 at a single dose of 3 mg/kg/d. These doses were chosen on

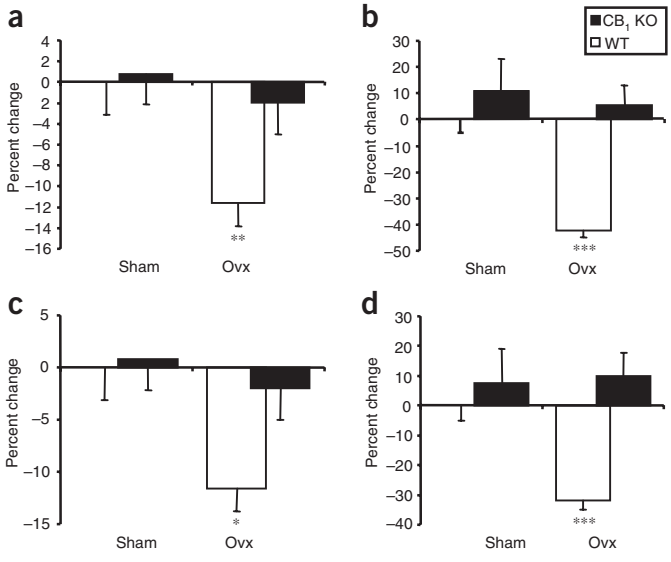

Figure 2 CB_1 knockout mice are protected against ovariectomy-induced bone loss. (**a**) Total BMD at the tibial metaphysis in CB_1 knockout (KO) mice and wild-type (WT) littermates before and after sham operation or ovariectomy (Ovx). (**b**) Bone volume/total volume assessed at the same site by microcomputed tomography. (**c**) Trabecular thickness assessed by microcomputed tomography. (**d**) Trabecular number assessed by µCT. Data are expressed as the percent change relative to the value in sham-operated wild-type animals and are mean + s.e.m. $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, comparing CB_1 knockout and wild-type mice.

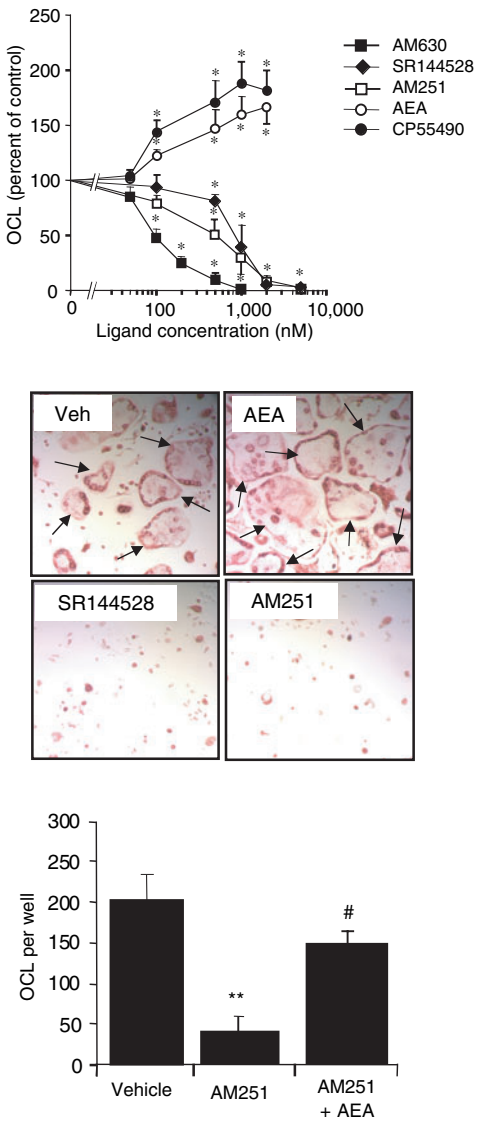

Figure 3 Regulation of osteoclast formation by cannabinoid receptor ligands. (**a**) Effects of the cannabinoid ligands on osteoclast formation in C57BL/6 bone marrow cultures stimulated with RANKL and M-CSF. The data shown are from three independent experiments and are expressed as a percent of values in control cultures. Significant inhibition or stimulation of osteoclast formation when compared with control is indicated by $^{*}P < 0.05$ or below. (**b**) Representative photomicrographs of vehicle-treated culture and cultures treated with anandamide (AEA), SR144528 and AM251 stained for TRAcP. Osteoclasts (indicated by arrows) were larger and more numerous in the AEA-treated cultures compared with vehicle-treated cultures and were virtually absent from the SR144528- and AM251-treated cultures. (**c**) Effect of AM251 alone (1 μM) and AM251 in combination with AEA (5 μM) on osteoclast formation. Data are presented as mean + s.d. $^{**}P < 0.001$ (vehicle versus AM251); $^{\#}P < 0.02$ (AM251 versus AM251 + AEA).

the basis of previous work, which has shown that AM251 and the related compound SR141716 prevent diet-induced obesity in wild-type mice in doses of 3–30 mg/kg/d[11–13]. We found that AM251 protected against ovariectomy-induced bone loss in a concentration-dependent manner at doses as low as 0.3 mg/kg/d (**Fig. 5a–c**). Bone histomorphometry showed that osteoclast numbers and active resorption surfaces were increased after ovariectomy in vehicle-treated animals, whereas no significant increase in either variable was observed in AM251-treated animals (**Supplementary Table 2** online). Osteoblast numbers were unaffected by AM251 treatment, indicating that the protective effect of cannabinoid receptor blockade on ovariectomy-induced bone loss was primarily mediated by inhibiting bone resorption rather than by stimulating bone formation. Administration of SR144528 to ovariectomized mice in a dose of 3 mg/kg/d also completely prevented ovariectomy-induced bone loss, with results almost identical to those observed with AM251 at 3 mg/kg/d (data not shown).

Effects of cannabinoid ligands on osteoclast apoptosis

We investigated the molecular mechanisms by which cannabinoid receptor blockade inhibits bone resorption, by studying the effects of cannabinoid receptor antagonists on apoptosis and key intracellular signaling pathways involved in osteoclast survival using purified rabbit osteoclasts and RANKL-generated mouse osteoclasts. We found that AM251 and SR144528 stimulated apoptosis of rabbit and mouse osteoclasts by 12–17-fold above control values (**Supplementary Fig. 2** online), as detected by deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) and typical changes in nuclear morphology on DAPI-stained cultures (**Supplementary Fig. 2** online). Activation of caspase 3 was observed in both rabbit and mouse osteoclasts that were exposed to cannabinoid antagonists (**Supplementary Fig. 2** online), and this was accompanied by a reduction in osteoclast numbers and inhibition of bone resorption (**Supplementary Fig. 2** online). The only considerable difference between the response of mouse and rabbit osteoclasts to cannabinoid antagonists was that maximal stimulation of apoptosis occurred at 2 μM in mouse osteoclasts compared with 10 μM in rabbit osteoclasts, presumably because of differences among the species in affinity of the receptors for the ligands tested. We also investigated the effects of AM251 on activation of key survival factors in osteoclasts, including extracellular signal–regulated kinase (ERK), the p65 component of NF-κB, NFATc1; c-jun and c-fos[14–16]. These studies showed that AM251 at a concentration of 2 μM abolished RANKL-induced ERK phosphorylation (**Fig. 6a**) and significantly inhibited RANKL-induced nuclear translocation and DNA binding of NFATc1, c-jun and c-fos ($P < 0.001$; **Fig. 6b–d**). In contrast, AM251 did not inhibit RANKL-induced NF-κB (p65) activation in this assay (data not shown).

DISCUSSION

The studies presented here indicate that the CB_1 receptor has a hitherto unrecognized role in the regulation of bone mass and bone loss resulting from estrogen deficiency. Adult CB_1 knockout mice had substantially higher BMD than wild-type littermates at several skeletal sites and CB_1 knockout mice were completely protected against ovariectomy-induced bone loss. The synthetic cannabinoid receptor antagonists AM251, SR144528 and AM630 inhibited osteoclast formation and bone resorption *in vitro*, and both AM251 and SR144528 protected against ovariectomy-induced bone loss *in vivo* by inhibiting osteoclastic bone resorption. Conversely, the endogenous cannabinoid agonist anadamide and the synthetic agonist CP55940 enhanced osteoclast formation *in vitro*, and anadamide reversed the inhibitory effects of AM251 osteoclast formation, consistent with a receptor-mediated effect. Osteoclasts generated from CB_1 knockout mice were resistant to the inhibitory effects of AM251 compared with wild-type mice, consistent with the hypothesis that the osteoclast inhibition we observed with cannabinoid antagonists was mediated, in part, by the CB_1 receptor. We also gained evidence to show CB_2 receptor–mediated osteoclast inhibition can also occur *in vitro*, from the

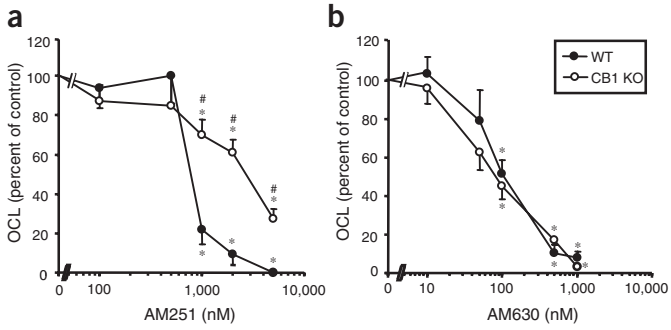

Figure 4 Osteoclasts (OCL) generated from CB_1 knockout mice are resistant to the inhibitory effects of CB_1-selective but not CB_2-selective receptor antagonists. (**a**) Effect of AM251 on osteoclast formation in RANKL- and M-CSF–stimulated bone marrow cultures prepared from CB_1 knockout mice and wild-type littermates. (**b**) Effect of AM630 on osteoclast formation in bone marrow mononuclear cells cultured from wild-type and CB_1 knockout mice. Data are presented as mean + s.d. $^{*}P < 0.05$ versus control cultures. $^{\#}P < 0.05$, CB_1 knockout versus wild-type cultures.

observation that the CB_2-selective antagonist AM630 inhibited osteoclast formation equally well in cultures prepared from CB_1 knockout and wild-type mice. The inhibitory effects on osteoclast formation that we observed at high AM251 concentrations (>1,000 nM) in CB_1 knockout cultures were probably attributable to blockade of the CB_2 receptor by this compound[10]. The extent to which CB_1 versus CB_2 receptors contributed to the prevention of the ovariectomy-induced bone loss that we observed with cannabinoid antagonist treatment *in vivo* remains to be determined, as does the relative contribution of central versus peripheral cannabinoid receptors in mediating this effect. Further work will also be required to determine whether the inhibitory effects we observed were the result of antagonism of the action of endogenous cannabinoid receptor ligands or to inverse agonism, given that AM630, AM251 and SR144528 have all been shown to elicit effects at cannabinoid receptors that are opposite to those produced by cannabinoid agonists, even in the absence of agonist binding[17].

The mechanism of osteoclast inhibition resulting from cannabinoid receptor blockade was probably the result of osteoclast apoptosis, because AM251 and SR144528 markedly enhanced apoptosis of mature rabbit osteoclasts and of RANKL-generated mouse osteoclasts. Exposure of mouse osteoclasts to AM251 blocked RANKL-induced ERK phosphorylation and prevented activation of several key osteoclast stimulatory factors including NFATc1, phosphorylated c-jun and c-fos. Notably, however, AM251 had no inhibitory effect on NF-κB activation, suggesting that cannabinoid receptor antagonists may cause osteoclast inhibition through the ERK-AP1 pathway rather than the NF-κB pathway.

The phenotype that we observed in CB_1 knockout mice indicates that the CB_1 receptor has an essential role in regulating BMD and bone loss that results from estrogen deficiency. Furthermore, the pharmacological studies *in vitro* show that the CB_1 and CB_2 receptors may have overlapping functions in the regulation of osteoclast activity. Further studies of the skeletal phenotype in CB_2 knockout mice will be required to fully evaluate the role of the CB_2 receptor subtype on bone metabolism and osteoclast function *in vivo*.

The results of our studies are important because we have shown that pharmacological antagonists of cannabinoid receptors represent a new class of osteoclast inhibitors, whereas cannabinoid receptor agonists act as stimulators of bone resorption. This suggests that cannabinoid receptor antagonists may represent a promising new class of antiresorptive drugs for the treatment of osteoporosis and other bone diseases associated with increased osteoclast activity. Our data also raise the possibility that recreational or therapeutic use of cannabis derivatives that act as agonists at cannabinoid receptors might enhance bone loss and predispose to osteoporosis.

METHODS

Cell culture. We generated mouse osteoclast cultures by isolating mononuclear cells from the bone marrow of mouse long bones, essentially as previously described[18]. The cells were cultured in αMEM with 10% FCS and M-CSF (100 ng/ml) for 3 d. We collected adherent cells and plated them at 0.5×10^4 cells/well in 96-well plates in 125 µl of αMEM supplemented with 10% FCS, M-CSF (25 ng/ml) and RANKL (100 ng/ml) for 4 d. We added test substances on day 7 and terminated the cultures on day 10. Rabbit osteoclast cultures were performed as described[19] and exposed to test substances for 48 h. We assessed osteoclast formation was assessed by counting tartrate-resistant acid phosphatase–positive multinucleated cells and bone resorption by measuring

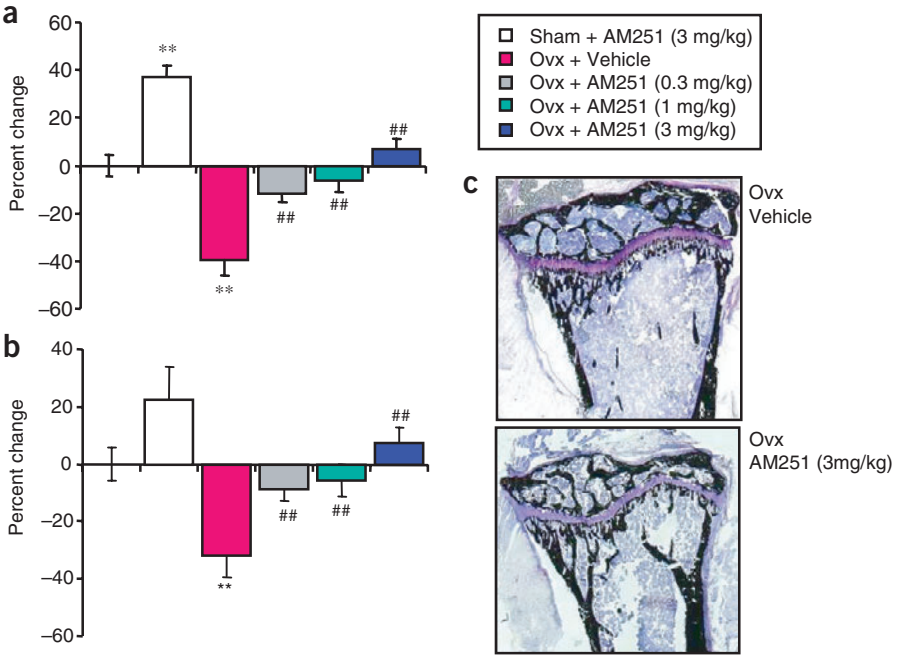

Figure 5 Cannabinoid receptor antagonists prevent ovariectomy-induced bone loss. (**a**) Effects of AM251 on trabecular bone volume (bone volume/total volume) in sham-operated (Sham) and ovariectomized (Ovx) C57BL/6 mice. (**b**) Effects of AM251 on trabecular number in sham-operated (Sham) and ovariectomized (Ovx) C57BL/6 mice. Data are expressed as percent change relative to sham-operated vehicle-treated control mice. (**c**) Representative photomicrograph of the tibial metaphysis 21 d after ovariectomy in a vehicle-treated mouse (top) and an AM251-treated mouse (bottom). Data are presented as mean ± s.e.m. $^{**}P < 0.001$, sham versus sham + AM251 and sham versus Ovx; $^{\#\#}P < 0.002$, Ovx versus Ovx + AM251.

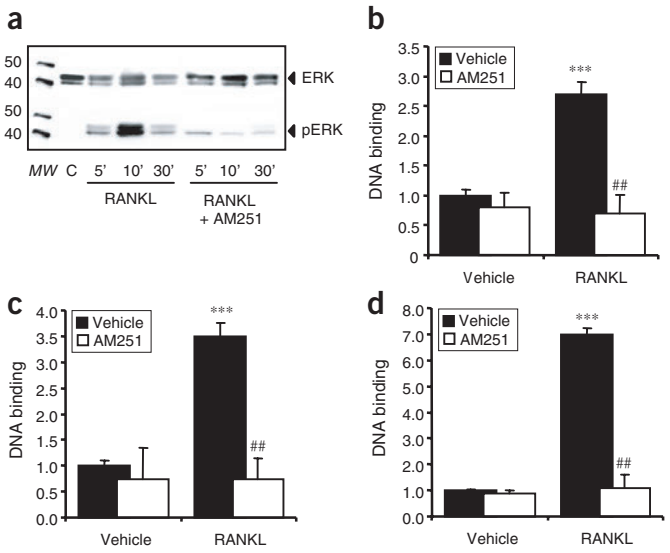

Figure 6 Cannabinoid receptor antagonists inhibit ERK, c-fos, c-jun and NFATc1 activation in mouse osteoclasts. (**a**) Effects of the cannabinoid antagonist AM251 (5 μM) on ERK phosphorylation as detected by western blot in vehicle (C)- and RANKL (100 ng/ml)-treated mouse osteoclasts. The numbers refer to the time (min) after RANKL stimulation. MW is molecular weight marker and the size of the marker bands is indicated in kDa. (**b**) Effects of AM251 on nuclear translocation and DNA binding of phosphorylated NFATc1 in mouse osteoclasts treated with RANKL (100 ng/ml) in the presence of vehicle or AM251 (2 μM). (**c**) Effects of AM251 on nuclear translocation and DNA binding of c-jun in mouse osteoclasts treated with RANKL (100 ng/ml) in the presence of vehicle or AM251 (2 μM). (**d**) Effects of AM251 on DNA binding of c-fos in mouse osteoclasts treated with RANKL (100 ng/ml) in the presence of vehicle or AM251 (2 μM). Values are expressed as the change relative to vehicle-treated cultures. Values in the bar charts are means + s.d. $^{***}P < 0.001$, vehicle versus RANKL-treated cultures; $^{\#\#}P < 0.001$, AM251- versus vehicle-treated cultures. The results shown are representative of three independent experiments.

resorption pit area using reflected light microscopy as described[20]. Apoptosis was detected by the characteristic changes in nuclear morphology after 4,6-diamidino-2-phenylinole (DAPI) staining as previously described[20], and by detection of fragmented DNA by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Intergen). We counted cells with clearly fragmented DNA or positive ApopTag labeling and expressed their number as a percentage of total cell number.

Immunofluorescence. We performed immunofluorescent staining of mouse osteoclasts for cannabinoid receptors using a rabbit antibody to the CB_1 receptor[21] (Caymen Chemical) and a rabbit antibody to the CB_2 receptor[22] (Caymen Chemical) followed by Alexa Fluor 488 goat antibody specific for rabbit IgG (Molecular Probes). Controls were performed by omitting the primary antibody. We identified osteoclasts by dual labeling with a mouse CD61-specific antibody directed against the vitronectin receptor (Pharmagen) followed by Alexa Fluor 594 goat antibody specific for mouse IgG.

Western blotting. We detected levels of intact (p-30) and activated (cleaved, p-19) caspase 3 by western blotting using a goat polyclonal antibody specific for caspase 3 (Santa Cruz), and a rabbit polyclonal antibody specific for cleaved caspase 3 (Cell Signalling Technology). We detected native and phosphorylated ERK by western blotting using antibodies to ERK and phosphorylated ERK (Cell Signalling Technology). We detected CB_1 and CB_2 receptors by western blotting using the same primary antibodies as for immunofluorescence, with an anti-rabbit secondary antibody linked to horseradish peroxidase (Caymen Chemical). We developed the chemiluminescent signal using SuperSignal (Pierce).

Molecular biology. We assessed activation and DNA binding of NFATc1, NF-κB (p65), phosphorylated c-jun and c-fos by analysis of nuclear extracts using TRANS-AM transcription factor assay kits (Active Motif). We detected CB_1 and CB_2 receptor mRNA with RT-PCR using Qiagen Quantitect custom primers for the CB_1 receptor were: 5′-CCTTGCAGATACAACCTT-3′ and 5′-TGCCATGTCTCCTTTGATA-3′; predicted product size, 94 bp. For the CB_2 receptor, they were: 5′-CATAAGCCGATCTCTCCAA-3′ and 5′-CCAAAGCTGGTGCAGGAA-3′; predicted product size, 82 bp. The PCR protocol consisted of an initial incubation at 95 °C for 15 min., followed by 40 cycles of 94 °C for 15 s, 56 °C for 30 s and 76 °C for 30 s. All reactions were run with no template and no reverse transcription controls.

Drugs. We dissolved all compounds in dimethylsulfoxide and added them to the cultures such that the final concentrations in all mixtures was 0.1%. We treated control cultures with dimethylsulfoxide alone at the same concentrations. AM251 and AM630 were obtained from Tocris. We synthesized SR144528 by coupling of 1-(4-methylbenzyl)-5-(4-chloro-3-methylphenyl)pyrazole-3-carboxylic acid chloride and (1S)-2-endo,exo-amino-1,3,3-trimethylbicyclo [2.2.1]heptane. We verified the identity and purity of SR144528 using nuclear magnetic resonance spectroscopy.

Animals. Animal experiments were approved by the ethical review board of the University of Aberdeen and were conducted in accordance with UK Home Office regulations. The strain of CB_1 knockout mice and wild-type littermates used in this study were as previously described[23]. We also performed experiments using C57BL/6 mice obtained from Harlan Laboratories. We performed ovariectomy or sham ovariectomy in 9-week-old adult female mice as previously described[24]. Treatment with AM251 and SR144528 was commenced 2 d after ovariectomy or sham ovariectomy by intraperitoneal administration of the drug in corn oil. Controls received corn oil alone. We continued the treatment for 19 d and terminated the experiment on day 21. We studied eight mice per group. Bone mineral density was measured by dual X-ray absorptiometry at the spine and femur using a PIXIMUS scanner; and at the tibial metaphysis by peripheral quantitative computed tomography using a Stratec Research M scanner or by microcomputed tomography, using a Skyscan 1072 scanner. After BMD scanning, we embedded the limbs and processed them for bone histomorphometry as previously described[24].

Statistics. Between-group comparisons were by ANOVA with Dunnet post-test. The concentrations at which 50% inhibition occurred were calculated from concentration response curves using the GraphPad PRISM computer program (GraphPad Software).

Note: Supplementary information is available on the Nature Medicine website.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

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