Cannabinoids and bone: endocannabinoids modulate human osteoclast function in vitro

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BACKGROUND AND PURPOSE
Both CB1 and CB2 cannabinoid receptors have been shown to play a role in bone metabolism. Crucially, previous studies have focussed on the effects of cannabinoid ligands in murine bone cells. This study aimed to investigate the effects of cannabinoids on human bone cells in vitro.

EXPERIMENTAL APPROACH
Quantitative RT-PCR was used to determine expression of cannabinoid receptors and liquid chromatography-electrospray ionization tandem mass spectrometry was used to determine the presence of endocannabinoids in human bone cells. The effect of cannabinoids on human osteoclast formation, polarization and resorption was determined by assessing the number of cells expressing \( \alpha_v \beta_3 \) or with F-actin rings, or measurement of resorption area.

KEY RESULTS
Human osteoclasts express both CB1 and CB2 receptors. CB2 expression was significantly higher in human monocytes compared to differentiated osteoclasts. Furthermore, the differentiation of human osteoclasts from monocytes was associated with a reduction in 2-AG levels and an increase in anandamide (AEA) levels. Treatment of osteoclasts with LPS significantly increased levels of AEA. Nanomolar concentrations of AEA and the synthetic agonists CP 55 940 and JWH015 stimulated human osteoclast polarization and resorption; these effects were attenuated in the presence of CB1 and/or CB2 antagonists.

CONCLUSIONS AND IMPLICATIONS
Low concentrations of cannabinoids activate human osteoclasts in vitro. There is a dynamic regulation of the expression of the CB2 receptor and the production of the endocannabinoids during the differentiation of human bone cells. These data suggest that small molecules modulating the endocannabinoid system could be important therapeutics in human bone disease.

INTRODUCTION
Mammalian tissues express at least two cannabinoid receptors, CB1 and CB2, both of which are G-protein coupled (Howlett et al., 2002; Alexander et al., 2011). Endogenous ligands (endocannabinoids) for these receptors also exist, prominent examples include arachidonyl ethanolamide (anandamide or AEA) and 2-arachidonoyl glycerol (2-AG). Both AEA and
2-AG are synthesized on demand, removed from their sites of action by tissue uptake processes and metabolized by intracellular enzymes (Pertwee and Ross, 2002). Together these lipids, enzymes and receptors constitute the endocannabinoid system. A clear role for the endocannabinoid system has been demonstrated in a variety of physiological processes including cardiovascular regulation, appetite control, pain processing, learning and memory, with evidence that the levels of endocannabinoids are altered in many pathophysiological situations (Di Marzo and Petrosino, 2007).

The endocannabinoid system is a recently identified therapeutic target in the control of bone mass. Recent studies have demonstrated abnormal bone phenotypes in mice lacking either CB1 or CB2 receptors; these phenotypes vary with age, gender or genetic background (for reviews see Bab et al., 2009; Idris and Ralston, 2010). Mouse bone cells and sympathetic nerve fibres that lie in close proximity to bone express cannabinoid receptors (Idris et al., 2005; Ofek et al., 2006; Tam et al., 2006; Bab et al., 2008). Importantly, the endocannabinoids 2-AG and AEA are produced in murine trabecular bone (Tam et al., 2008) and human osteoclasts (Rossi et al., 2009).

Synthetic cannabinoid receptor agonists and inverse agonists have also been shown to reduce bone loss in mice following ovariectomy and to have direct effects on mouse bone resorbing cells (osteoclasts) and bone forming cells (osteoblasts) in vitro (Idris et al., 2005; Ofek et al., 2006; Tam et al., 2006; Idris, 2008; Idris et al., 2009; Sophocleous et al., 2011).

In this study we further investigated the role of cannabinoids in bone physiology by addressing a key question that has been little investigated; what effect do cannabinoids have on the function of human bone cells in vitro? In the light of recent reports demonstrating significant associations between CB2 receptor genotypes and osteoporosis in humans (Karsak et al., 2005), it is crucial to understand the pharmacology of the cannabinoids in human bone cells. Previous in vitro studies have primarily focussed on the effects of cannabinoid ligands in murine bone cells (Bab et al., 2009). There is a possibility that the effects of cannabinoid ligands may be subject to species differences and the compounds may have distinct profiles in mouse and human bone cells. It is well known that GPCR agonists can display divergent effects on bone cells obtained from mouse compared to human cells, this is exemplified in the effects of PGE2 (Take et al., 2005). Only one study (Rossi et al., 2009) has examined the effect of only one CB2 receptor antagonist on human osteoclast formation; there are no data in the current literature on the effects of cannabinoids on human osteoclast function.

Our results provide important new insights into the dynamic regulation of the endocannabinoid system in human osteoclast differentiation and demonstrate effects of cannabinoid agonists on human bone resorbing cells in vitro. This study therefore adds to the evidence, primarily obtained from murine cells in vitro, that modulation of CB2/CB2 may be an approach to the treatment of diseases caused by excessive osteoclast activity such as osteoporosis.

**Methods**

Cell culture reagents and chemicals were purchased from Sigma Aldrich Company Ltd (UK) unless otherwise stated. Cannabinoid compounds 2-AG, AEA, CP 55,940 [- (1-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol], JWH 015 [- (2-methyl-1-propyl)-1H-indol-3-yl)-1-naphthalenemethanone], AM251 [- N-(piperidin-1-yl)-5-(4-iodo-phenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide], AM630 [- 6-iodo-2-methyl-1-[2(4-morpholinyl)ethy]-1H-indol-3-yl][4-methoxyphenyl)methanone] were from Tocris Cookson Ltd (Bristol, UK), and SR141716A [- N-piperidino-5-(4-chlorophenyl)-1(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide] and SR144528 [- N-[[1S]-endo-1,3,3-trimethyl bicyclo [2.2.1] heptan-2-yl]-5-[4-chloro-3-methylphenyl]-1-[4-(methylbenzyl)-pyrazole-3-carboxamide] were from Sanofi-Aventis (Montpellier, France). URB-597 (cyclohexylcarbamic acid 3’carbamoyl-biphenyl-3-yl ester) was purchased from Biomol International (USA). All compounds were dissolved in dimethyl sulfoxide (DMSO) such that the final concentration of DMSO did not exceed 0.1%.

**Cell culture**

All cells, unless otherwise stated, were cultured at 37°C/5% CO2 in α-modified minimal essential media (α-MEM) containing 10% (v/v) fetal calf serum (FCS), 2% antibiotic mixture (PS; 100 IU·mL-1 penicillin, 100 μg·mL-1 streptomycin) and 2 mM·L-glutamine. CB2 stably transfected CHO cells (Ross et al., 1999) and mouse brain membranes (Price et al., 2005) were prepared as previously described.

**Isolation of osteoblasts**

Mouse calvarial osteoblasts were isolated from the calvarial bones of 2-day-old C57BL/6 mice by sequential collagenase digestion and grown to confluence as previously described (Armour et al., 2001).

**Peripheral blood mononuclear cell isolation and osteoclast generation**

Human osteoclasts were generated from peripheral blood mononuclear cells (PBMCs) donated with informed consent from healthy volunteers (with approval from the North of Scotland Research Ethics Committee) as previously described (Whyte et al., 2009). In brief, PBMCs were isolated by centrifugation over Lymphoprep (Axis-Shield Diagnostics Ltd) and seeded into 75 cm2 flasks in culture medium supplemented with 20 ng·mL-1 macrophage colony stimulating factor (M-CSF) (R&D Systems Europe Ltd) to allow adherence and expansion of M-CSF-dependent monocyes (approximately 7 days). To generate osteoclasts, the highly enriched M-CSF-dependent monocyes were harvested by trypsinization and gentle scraping, and then re-plated at a seeding density of 200 000 cells·mL-1 in medium containing 20 ng·mL-1 M-CSF and 100 ng·mL-1 receptor activator of NF-kB ligand (RANKL, Peprotech EC Ltd). Medium was refreshed every 2–3 days and after approximately 5–7 days in RANKL more than 80% of cells were αaβ3-positive with numerous multinucleated osteoclasts.

**Analysis of human osteoclast formation**

To study the effect of cannabinoids on osteoclast formation, osteoclasts were generated on plastic 96-well plates as described above, in the presence of test compounds or vehicle
(0.1% v/v DMSO). Compounds were added to highly enriched M-CSF-dependent monocytes at the same time as the RANKL and were present throughout the duration of the culture. Osteoclasts generated in the presence of 2-AG, AEA or vehicle were cultured in medium containing 2% FCS rather than 10% FCS in order to reduce the binding of these lipophilic compounds to serum. This reduction in serum levels had no detrimental effect on cell viability or osteoclast formation. Once formed, total cell viability was determined using the Alamar Blue assay as described by Taylor et al. (2007). Cells were then washed thoroughly in HBSS before being fixed with 4% paraformaldehyde (v/v). Osteoclasts were stained with 23c6 anti-α,β3 (Serotec) and Alexa Fluor 488 goat anti-mouse antibodies to quantify total α,β3 fluorescence as a measure of osteoclast formation using a BioTek FL600 plate reader. Total α,β3 immunofluorescence showed a significant correlation with the total number of α,β3-positive multinucleated cells (Figure S1).

**Analysis of human osteoclast polarization and resorption**

Human osteoclasts were generated on 5 mm diameter elephant ivory dentine discs in 96-well plates and treated with test compounds at the first sign of osteoclast resorption (approximately day 7 of RANKL treatment). Upon addition of AEA or vehicle at the first signs of resorption, cultures were switched from 10% FCS to 2% FCS for reasons described above; cultures treated with CP 55 940 or JWH015 were maintained at 10% FCS. Cultures were terminated 3–5 days later by fixing in 4% paraformaldehyde. Intracellular F-actin was visualized by staining with 0.5 μg·ml−1 tetramethylrhodamine isothiocyanate (TRITC)-phalloidin as previously described (Coxon et al., 2001) and the number of F-actin rings per disc was counted. To assess osteoclast-mediated resorption, cells were removed from the discs and pits in the mineral surface were visualized by reflected light microscopy. The area of resorbed dentine was examined using a Zeiss Axiolab reflective light microscope and quantified using software developed in-house based on Aphelion (ADCIS, France) ActiveX components.

**Quantitative PCR**

RNA extraction and cDNA synthesis were carried out as described previously (Idris et al., 2005). RNA was extracted from M-CSF-dependent monocytes and mature osteoclasts (after 7 days in RANKL). The expression of the human CB₁ and CB₂ receptors and the control gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real time PCR using FAM- and VIC-labelled TaqMan probes (Applied Biosystems, UK, CB₁- Hs00275634_m1 FAM and CB₂- Hs00275635_m1, VIC-GAPDH) and using a DNA Engine Opticon 2 Real Time Cycler (MJ Research). Control reactions with no template and no reverse transcriptase were run on each occasion. Reactions were carried out at 95°C for 15 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min.

**Immunocytochemistry**

Human osteoclasts and mouse osteoblasts were fixed with 4% paraformaldehyde in PBS, blocked using 10% FCS for 10 min and stained using an antibody to CB₁ (Abcam) or CB₂ (Affinity Bioreagents) followed by Alex Fluor goat anti-rabbit 488 (Invitrogen). Nuclei were counterstained with 0.5 μM TO-PRO-3 iodide (Invitrogen). Control cells were stained with normal rabbit IgG (Santa Cruz). Cells were examined on a Zeiss LSM510 Meta Confocal microscope and images captured using LSM image capture software.

**Western blotting**

Mouse osteoblasts, mouse brain homogenates, CHO cells stably transfected with CB₁, human monocytes and human osteoclasts (isolated at three time points during the course of differentiation), were lysed in RIPA buffer [PBS, 0.5% (w/v) sodium dodecyl sulphate, 0.5% (w/v) sodium deoxycholate and 1% (v/v) Igepal CA-630] containing Protease Inhibitor Cocktail (Sigma). The protein concentration of the lysates was determined (BCA assay, Pierce) and equal quantities (50 μg) of denatured samples were electrophoresed on pre-cast Bis-Tris 12% criterion gels (Bio-Rad, USA) and transferred to polyvinyl difluoride (PVDF) membranes by semi-dry transfer [Bio-Rad Trans-blot SD Semi-Dry Transfer cell plates (Bio-Rad, USA)]. Membranes were blocked for 1 h by using Odyssey blocking buffer (LI-COR, USA) diluted 1:1 in Tris buffered saline (TBS), then incubated with primary antibodies for CB₁ or CB₂ overnight at 4°C in blocking buffer, prepared as outline above, containing 0.1% Tween (TBST). The following day membranes were washed with TBST and then incubated with infrared-labelled secondary antibodies for 1 h in the dark followed by further wash steps, the final of which was in TBS. Blots were visualized using a LI-COR Odyssey Infrared Imager.

**Analysis of Rho activation in human osteoclasts**

Human osteoclasts were deprived of FCS, RANKL and M-CSF overnight and then treated for 2.5 or 5 min with α-MEM alone (control), α-MEM containing 10% FCS or 1 mg·mL−1 BSA, vh (0.1% DMSO) or 1 μM CP 55 940. Quantification of Rho-GTP in 0.5 mL of cleared cell extract (harvested from two wells of a six-well plate) was performed using a Rhotekin pull-down assay according to the manufactures instructions (Upstate) as previously described (Whyte et al., 2009). Treatments with GTPγS (positive control) and GDP (negative control) ensured that the pull-down procedures were working properly. Total Rho was determined from cell extracts not subject to pull-down in order to ensure equal protein quantities between samples.

**LC-MS/MS analysis**

M-CSF-dependent monocytes, mature osteoclasts (after 7 days in RANKL) (9.5 cm² wells) and cell lines at 70–90% confluence (75 cm² flasks) were washed and scraped into PBS and collected by centrifugation at 548 x g for 10 min at 4°C. The pellet was resuspended in 50/50 methanol/acetoniitrile containing 6 pmol d4-AEA (internal standard) (QMX Laboratories Ltd) and homogenized by sonication. The sonicated mixture was made up to 70% water and centrifuged to remove cell debris. The supernatant was applied to a preconditioned Strata-X SPE cartridge (Phenomenex), washed twice, eluted in 100% methanol and evaporated to dryness under nitrogen. The residue was resuspended in 50 μL mobile phase. Standards containing AEA (0.01–2 pmol...
Endocannabinoids are produced by human osteoclasts, osteoblast-like cells and mouse osteocytes

Basal levels of 2-AG and AEA were measured in primary human monocytes and osteoclasts. 2-AG was detected in both human monocytes and osteoclasts (Figure 2A), with significantly lower levels in osteoclasts compared with monocytes (monocytes -0.43 ± 0.14 nmol·mg⁻¹·protein; osteoclasts 0.11 ± 0.02 nmol·mg⁻¹·protein). 2-AG was also detected in the mouse osteocyte-like cell line MLO-Y4 (0.83 ± 0.11 nmol·mg⁻¹·protein) and two human osteoblast-like cell lines, MG-63 (0.11 ± 0.02 nmol·mg⁻¹·protein) and HOS TE85 (0.28 ± 0.03 nmol·mg⁻¹·protein). AEA was not detected in human monocytes but present in osteoclasts (0.13 ± 0.02 nmol·mg⁻¹·protein) (Figure 2B). AEA was also detected in MG-63 cells (0.12 ± 0.01 nmol·mg⁻¹·protein), MLO-Y4 cells (0.10 ± 0.01 nmol·mg⁻¹·protein) but not in HOS TE85 cells.

Treatment with LPS did not alter the levels of 2-AG detected in human osteoclasts from three separate donors (0.06 ± 0.01 to 0.09 ± 0.01 nmol·mg⁻¹·protein, 0.24 ± 0.08 to 0.20 ± 0.02 nmol·mg⁻¹·protein, 0.19 ± 0.04 to 0.12 ± 0.04 nmol·mg⁻¹·protein, Figure 2C). However, treatment of human osteoclasts with LPS did increase the amount of AEA detected (0.06 ± 0.01 to 0.14 ± 0.03 nmol·mg⁻¹·protein, not detected to 0.15 ± 0.09 nmol·mg⁻¹·protein or 0.22 ± 0.07 nmol·mg⁻¹·protein, Figure 2D).

Endocannabinoids inhibit human osteoclast formation at micromolar concentrations

The detection of both cannabinoid receptor subtypes together with the presence of endocannabinoids in human monocytes and fully differentiated osteoclasts suggests that cannabinoid receptor ligands may have direct effects on the differentiation of osteoclasts. To determine the effect of endocannabinoids on human osteoclastogenesis, highly enriched M-CSF-dependent human mononuclear cells were stimulated to differentiate into multinucleated osteoclasts, capable of functional resorption, by the addition of M-CSF and RANKL in the continual presence of 1 nM–1 μM AEA or 2-AG. Osteoclast formation was significantly reduced in the presence of 10 μM AEA (Figure 3A) or 10 μM 2-AG (Figure 3B) to 36 ± 3% and 42 ± 4% of control, respectively. Cell viability was not altered in the presence of AEA or 2-AG; therefore, the inhibition of osteoclastogenesis was caused by an attenuation of differentiation rather than a decrease in cell number caused by toxicity (Figure S2A and B).

In order to determine the receptor responsible for the endocannabinoid-induced inhibition of osteoclastogenesis, selective cannabinoid antagonists were utilized with the purpose of selectively blocking the CB1 (100 nM AM251), CB2 (100 nM AM630) or TRPV1 (1 μM capsazepine) receptors. At the concentration tested, none of the antagonists significantly affected osteoclast formation alone, and when added to cultures containing 10 μM AEA or 2-AG the antagonists were incapable of reversing the inhibitory effects of the endocannabinoids (Figure 3C). These results suggest that the endocannabinoid-mediated inhibition of osteoclast differentiation is independent of CB1, CB2 or TRPV1 receptor activa-
tion and is mediated by another receptor, such as the putative cannabinoid receptor GPR55, or is a non-receptor mediated effect.

**CP 55 940 inhibits osteoclast formation – CB2 mediated**

Similar to the effects of endogenous cannabinoids on human osteoclast formation, the synthetic non-selective CB1/CB2 agonist CP 55 940 did not significantly affect osteoclast formation at concentrations from 1 nM to 100 nM; however, 1 μM CP 55 940 significantly reduced osteoclast formation as assessed by measurement of VNR fluorescence (Figure 4A). This decrease in osteoclast formation was not associated with any significant reduction in cell viability (Figure S2C). Human osteoclast formation was not significantly affected in the presence of the CB2-selective agonist JWH015 (Figure 4B); however, although not significant, JWH015 showed a trend towards an inhibition of osteoclast formation whereby at

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**Figure 1**

Cannabinoid receptor expression in human monocytes, osteoclasts and mouse osteoblasts. Quantitative real time PCR was performed using a primer Taqman probe set specific for CB1 (A) or CB2 (B) on isolated RNA from human M-CSF-dependent monocytes and differentiated osteoclasts formed in the presence of RANKL and M-CSF. CB1 and CB2 mRNA levels were normalized to GAPDH (housekeeping gene – levels remained constant during osteoclast differentiation). Results reported as fold change in gene expression relative to untreated cells after normalization against GAPDH. Mean ± SEM; n = 4 experiments – levels measured in triplicate for each donor. ***P < 0.001 Student’s t-test. CB1 and CB2 protein expression in human monocytes, osteoclasts and mouse osteoblasts was detected by Western blotting. Cell lysates were prepared and equal amounts of protein were electrophoresed on polyacrylamide-SDS gels and immobilized to PVDF membranes by Western blotting. (C) CB1 expression is shown in mouse brain homogenates (brain), M-CSF-dependent monocytes (mono) and osteoclasts formed in the presence of RANKL isolated at days 2, 5 and 6 (note the glycosylated form of CB1 is 64 kDa and the non-glycosylated form is 54 kDa) and mouse calvarial osteoblasts (oblast). (D) CB2 expression is shown in CB2 transfected CHO cells (CB2TCHO), monocytes, osteoclasts formed in the presence of RANKL isolated at days 3, 4 and 5 and mouse osteoblasts. β-Actin served as a loading control. Results shown are representative of three experiments. Immunocytochemical detection of CB1 and CB2 in human osteoclasts (E) and mouse osteoblasts (F) by immunofluorescence staining – CB1 or CB2 in green with a nuclear counterstain in either blue (osteoclasts) or red (osteoblasts). Cells were visualized by confocal microscopy. Negative controls were stained with normal rabbit IgG (middle panel). Bar = 20 μm.
1 μM VNR fluorescence was reduced to 76.9% of control, again this was not associated with a reduction in cell viability. This may have reached statistical significance at higher concentrations but in order to retain CB2 receptor selectivity higher concentrations were not used.

To determine the receptor responsible for the modest inhibitory effect of CP 55 940 on human osteoclast formation, M-CSF-dependent mouse bone marrow macrophages from CB1−/−, CB2−/− and GPR55−/− mice were stimulated to differentiate into TRAP-positive multinucleated osteoclasts by the addition of M-CSF and RANKL in the presence of either vehicle or 1 nM–1 μM CP 55 940. In wild-type cultures 100 nM–1 μM CP 55 940 significantly decreased mouse osteoclast formation relative to control (Figure 4C). Likewise, in CB1−/− and GPR55−/− cultures, CP 55 940 significantly decreased mouse osteoclast formation relative to control as determined by a reduction in the number of TRAP-positive osteoclasts with three or more nuclei. The inhibitory effect of CP 55 940 on mouse osteoclast formation was in fact significantly augmented in CB1−/− cultures, further ruling out the possibility that CB1 mediates the inhibitory response on osteoclast formation induced by CP 55 940. Notably, in wild-

Figure 2
Endocannabinoids are produced by human osteoclasts – levels change during osteoclast differentiation and are influenced by LPS. (A) Detection of 2-AG in human monocytes (n = 15 from six separate donors) and osteoclasts (n = 16 from six separate donors). Levels in cell extracts were normalized to mg−1 of protein. (B) Detection of AEA in the same samples as in (A). Values represent the mean ± SEM *P < 0.05, Student’s unpaired t-test. ND, not detected. Levels of 2-AG (C) and AEA (D) in human osteoclasts following LPS treatment. Cells were washed in PBS and treated for 90 min with 200 μg·mL−1 LPS. Values represent the mean ± SEM from three separate donors. Values represent the mean ± SEM **P < 0.01, Student’s unpaired t-test. ns, not significant.
type, CB$_2^{-/-}$ and GPR55$^{-/-}$ cultures monocytes still appeared to differentiate into TRAP-positive mononuclear cells in the presence of CP 55 940. In CB$_2^{-/-}$ cultures the inhibitory effect of CP 55 940 was absent, implicating CB$_2$ as the receptor mediating the inhibition of mouse osteoclast formation induced by CP 55 940 in vitro – representative images shown in Figure 4D.

In light of the inhibitory effect of CP 55 940 on osteoclast formation, we also examined the effect of cannabinoid receptor antagonists on human osteoclast formation. The CB$_2$ cannabinoid receptor antagonists SR144528 and AM630 together with the CB$_1$ receptor antagonist SR141716A did not significantly affect human osteoclast formation; however, the CB$_1$ antagonist AM251 at 1 µM significantly increased human osteoclast formation relative to control, albeit a modest increase to 134% of control (Figure S3).

**AEA stimulates human osteoclast polarization and resorption – CB$_2$ mediated**

The effect of the endocannabinoids on the polarization and resorptive activity of mature human osteoclasts was investigated by the addition of AEA to cultures after osteoclasts had formed on dentine discs in the absence of drugs, in order to mitigate any potential effects on formation. Cultures were terminated approximately 5 days later by fixing with 4% paraformaldehyde after which F-actin ring number, osteo-

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**Figure 3**

The inhibition of human osteoclast differentiation induced by 10 µM AEA or 2-AG is not mediated by CB$_1$, CB$_2$ or TRPV1 receptors. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of RANKL and 1 nM–10 µM AEA (A) or 2-AG (B) for 7 days. (C) Osteoclasts were cultured, as described above, in the presence of 10 µM AEA or 2-AG together with 100 nM AM251, 100 nM AM630 or 1 µM capsazepine. Cells were fixed and stained for the vitronectin receptor and immunofluorescence intensity was measured and expressed relative to control cultures as an indication of osteoclast number. Mean ± SEM; n = 3–4 experiments – 4–5 replicates each. **P < 0.01 compared with control; one-way ANOVA with Dunnett’s post-test.
clast number and resorption pit area were quantified. F-actin rings are a characteristic cytoskeletal feature of polarized, actively resorbing osteoclasts. The formation of an F-actin ring is essential for osteoclast function and is a quantifiable measure of osteoclast activity. Treatment of human osteoclasts with 100 nM AEA resulted in an increase in the number of F-actin rings to 168/11006 22% of control (Figure 5A) and resorption pit area to 390/11006 100% of control (Figure 5B) – representative images of resorption pits in dentine shown in Figure 5C. Collectively, these data show that the endocannabinoid AEA significantly enhances osteoclast function. Interestingly, treatment of human osteoclasts with AEA in the presence of an inhibitor (URB-597) of FAAH, the major enzyme for AEA catabolism, did not significantly augment the stimulatory effect on osteoclast polarization and resorption seen in the presence of AEA alone (Figure 5D). To determine the receptor implicated in the stimulating effects of AEA in human osteoclasts, similar experiments were performed in the presence of the CB1 and CB2 antagonists SR141716A and SR144528, respectively. A significant attenuation of the stimulatory effect of AEA in the presence of SR144528 compared to treatment with 100 nM AEA alone (Figure 5E) suggests that the effect of AEA on human osteoclast function was mediated by CB2 receptors.

The effect of 2-AG on human osteoclast activity was also investigated. However, there was a high level of inter-donor

Figure 4
CP 55 940 inhibition of human and mouse osteoclast differentiation is mediated by CB2 receptors. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of 1 nM–10 μM CP 55 940 (A) or JWH015 (B) for 7 days, assayed for cell viability and then fixed and stained for VNR to quantify osteoclast number. Immunofluorescence intensity was measured and expressed relative to control cultures as an indication of osteoclast number. Mean ± SEM; n = 6–7 experiments (CP 55 940) and 4 experiments (JWH015) – 5 replicates each. ***P < 0.001 one-way ANOVA with Bonferroni post-test. (C) M-CSF-dependent bone marrow macrophages from wild-type (WT), CB1-/-, CB2-/- or GPR55-/- mice were cultured in M-CSF and RANKL in the presence of vehicle or 1 nM–1 μM CP 55 940 for 5 days and then fixed and stained for TRAP. The number of TRAP-positive multinucleated osteoclasts were counted and expressed as a percentage of control. Mean ± SEM; n = 3–4 experiments – 5 replicates each. *P < 0.05, **P < 0.01, ***P < 0.001 CP 55 940 compared to control and #P < 0.05, ##P < 0.01, ###P < 0.001 compared with WT – one-way ANOVA with Bonferroni multiple comparison post-test. (D) Representative images of TRAP positive, multinucleated mouse osteoclasts – note the lack of inhibition of osteoclast formation in the presence of CP 55 940 in CB2-/- cultures.
variability in the results, preventing accurate analysis of the effect of this endocannabinoid.

The synthetic cannabinoid CP 55 940 stimulates human osteoclast polarization and resorption – mediated by CB₁/CB₂

Consistent with the effects seen with the endogenous cannabinoid AEA, treatment of human osteoclasts with the synthetic agonist CP 55 940 resulted in a significant increase in the number of F-actin rings (with 100 nM and 1 μM CP 55 940) (Figure 6A) and resorption area (with 1 nM–1 μM CP 55 940) relative to control (Figure 6B) – representative images of actin rings and resorption pits in dentine shown in Figure 6C. Treatment of cultures with 1 μM CP 55 940 in the presence of 100 nM SR141716A or SR144528 resulted in a significant reversal of the increases in F-actin ring number (79% and 82% reduction, respectively) and resorption area (80% and 78% reduction, respectively) seen after treatment with 1 μM CP 55 940 alone (Figure 6D).
The synthetic cannabinoid CP 55 940 stimulates Rho activation in human osteoclasts

Having established an increase in osteoclast polarization and activity with CP 55 940, we next investigated potential signalling responses that may be involved in eliciting these responses. The small GTPase Rho is known to play a role in cytoskeletal arrangement and osteoclast resorption (Chelalaiah, 2005). Using a pull-down assay, we showed that treatment of human osteoclasts with CP 55 940 for 2.5 or 5 min caused an increase in levels of active GTP-bound Rho relative to control (Figure 6E).
The CB2-selective agonist JWH015 stimulates human osteoclast polarization and resorption
Consistent with the effects seen with CP 55 940 and AEA, treatment of human osteoclasts with the CB2-selective agonist JWH015 increased osteoclast actin ring formation and resorption. Treatment with 100 nM JWH015 caused a highly significant increase in the number of actin rings to 214.5 ± 42% of control (Figure 7A) and resorption area to 279 ± 75% of control (Figure 7B) further supporting a role for CB2 receptor activation stimulating osteoclast function.

Discussion and conclusions
We and others have recently shown that the CB1 and/or CB2 receptors are expressed on mouse osteoclasts and osteoblasts (Idris et al., 2005; Ofek et al., 2006; Tam et al., 2006). In this study, both receptors were detected at the mRNA level in human monocytes and fully differentiated osteoclasts, as previously described by Galiegue et al. (1995) and Rossi et al. (2009), respectively. Here we report for the first time a significant decrease in CB2 mRNA levels during osteoclast differentiation; no such changes were seen in CB1 mRNA expression during osteoclast differentiation. In line with our findings in human osteoclasts, previous studies have demonstrated that down-regulation of CB2 is necessary for successful differentiation of B cells (Carayon et al., 1998) and of myeloid precursors into neutrophils (Jorda et al., 2003). It would appear that the down-regulation of CB2 is also necessary for the initiation of osteoclast differentiation.

N-linked glycosylation is the most common post-translational modification of GPCRs that correlates to the localization and function of receptors. Studies in the rat brain have shown that the CB1 receptor contains three glycosylation sites, two of which are actually glycosylated. The majority of CB1 receptors found in the brain are in the mature glycosylated form (65–80%). The molecular weight of mature glycosylated CB1 is 64 kDa (Song and Howlett, 1995), this is in line with the band detected in both osteoblasts and osteoclasts by Western blotting in this study. The predicted molecular weight of the non-glycosylated CB1 receptor is 53 kDa (Pettit et al., 1998). The non-glycosylated form is thought to be produced as a result of the receptor either escaping co-translational glycosylation or being trimmed of carbohydrate chains by exo- and endoglycosides, thus different cells with differences in their carbohydrate processing may give rise to different molecular weight species producing a shift in size on a Western blot and this may account for the different molecular weight bands consistently produced when probing for CB1 by Western blotting in bone cells in this study. Here we have shown that osteoclasts and osteoblasts appear to express CB1 mainly in the non-glycosylated form (stronger 53 kDa band compared with 63 kDa band), whereas the brain, as has been shown by others, contains high levels of the glycosylated 64 kDa form and lower expression of the non-glycosylated/partially glycosylated forms (Song and Howlett, 1995). The role of CB1 receptor glycosylation in ligand binding, activation and trafficking is not known (Song and Howlett, 1995) and as such the relevance of this finding in bone cells remains to be determined.

Previous studies have detected AEA and 2-AG in human osteoclasts (Rossi et al., 2009), whole bone (Tam et al., 2008) and mouse osteoblast-like cells (Bab et al., 2008). Using LC-MS/MS, we demonstrate the basal production of 2-AG and AEA in human monocytes and osteoclasts, with 2-AG levels decreasing, and AEA levels increasing during the differentia-
JWH015 (CB2-selective agonist) stimulated human osteoclast decrease in CB2 agonism/expression, which necessarily osteoclasts express both FAAH and NAPE-PLD (Rossi added compound. In line with these observations, human osteoclasts (Figure 1) may preclude an effect of exogenously Indeed, the high levels of endogenous AEA present in mature that is not readily modulated by exogenously added agonists. Indeed, the high levels of endogenous AEA present in mature osteoclasts (Figure 1) may preclude an effect of exogenously added compound. In line with these observations, human osteoclasts express both FAAH and NAPE-PLD (Rossi et al., 2009). Interestingly, the CB2 receptor antagonist, AM630 at nM concentrations of CP 55 940 significantly inhibit osteoclast formation and in CB1/CB2 agonist) and CP 55 940 (non-selective CB1/CB2 agonist) and JWH015 (CB2-selective agonist) stimulated human osteoclast polarization and resorption. The stimulating effect of CP 55 940 on osteoclast polarization and resorption was attenuated by both the CB1-selective antagonist SR141716A and the CB2-selective antagonist SR144528, possibly implicating a role for both CB1 and CB2 receptors. In this study, the levels of active, GTP-bound Rho were investigated in osteoclasts by use of a Rho pull-down assay whereby CP 55 940 significantly increased the levels of GTP-bound Rho compared with vehicle control. Similarly, the endogenous cannabinoid AEA, levels of which are increased in mature osteoclasts (Figure 2), stimulates human osteoclast polarization and resorption at nM concentrations. The increase in osteoclast polarization in the presence of AEA is consistent with its known ability to stimulate pathways inducing actin polymerization (Gokoh et al., 2005), involving c-src and Rho GTPase, which are also known to be essential for osteoclast function (Saltel et al., 2004). The stimulating effect of AEA appears to be CB2-receptor mediated. It is also notable that the pharmacological profile of the antagonists used is such that they are receptor subtype selective but not entirely specific; both compounds have affinity for CB1 and CB2 receptors and may interact with novel cannabinoid receptors (Ryberg et al., 2007). Indeed, we have demonstrated a role of GPR55 in osteoclast physiology (Whyte et al., 2009). While AEA had a robust, reproducible effect on human osteoclasts function, the effect of 2-AG could not be clearly established in this study; there was a high level of inter-donor variability in the results, preventing accurate analysis of the effect of this endocannabinoid. This variability may indeed reflect the observation of CB2 single nucleotide polymorphisms known to affect bone physiology in humans and is the subject of ongoing investigations (Karsak et al., 2005; 2009; Yamada et al., 2007). It is also notable that the inhibition of osteoclast formation observed at 10 μM 2-AG (Figure 3) is not subject to inter-donor variability, perhaps further evidence of the non-receptor mediated nature of this effect.

Our finding that LPS increased the production of osteoclast-stimulating AEA from osteoclasts, showing that the levels of these endogenous ligands are subject to modulation by known osteogenic factors, supports the relevance of endocannabinoid production by bone cells and its local effect on osteoclasts. LPS is a major bacterial endotoxin that stimulates the production of 2-AG and/or AEA in macrophages (Varga et al., 1998; Di Marzo et al., 1999; Liu et al., 2003). We found that treatment of human osteoclasts with LPS caused an increase in the levels of AEA but did not cause any detectable change in 2-AG levels. LPS is a potent activator of osteoclasts and promotes osteoclast survival (Suda et al., 2002). Given that LPS has been shown to cause inflammatory bone loss in diseases characterized by increased osteoclast activity, such as periodontal disease (Nair et al., 1996; Abu-Amer et al., 1997), it is possible that this may be due in part to the local production of AEA, causing an autocrine stimulation of osteoclastic bone resorption.

In conclusion, in this study we demonstrated a dynamic regulation of the expression of cannabinoid receptors and production of endocannabinoids during human osteoclast differentiation. Crucially we demonstrated for the first time that cannabinoid agonists have profound effects on human osteoclast function in vitro; both synthetic and endogenous cannabinoids significantly increased human osteoclast polarization and resorption and the stimulating effect of AEA on human osteoclast resorption was attenuated by CB2 receptor antagonists. Furthermore, as pro-inflammatory agents such as LPS can modulate endocannabinoid production, our find-
ings suggest that these endogenous lipid mediators may exert a local influence on bone turnover by stimulating bone resorption particularly during inflammation. It is notable that CB2 \(^{−/−}\) mice display age-related bone loss. Our data suggest that, in contrast to observations in mouse, cannabinoids do not affect human osteoclast formation at physiologically relevant concentrations, but rather stimulate human osteoclast activation. Hence, there may be important species differences in the role and function of CB receptors in bone physiology. Our observations are also important in the context of the previous demonstrations that there are significant associations between CB2 receptor genotypes and osteoporosis in humans (Karsak et al., 2005; 2009; Yamada et al., 2007). The consequences of these polymorphisms to either the under/over activation or expression of the CB2 receptor have yet to be elucidated. Our findings indicate that small molecules targeting the endocannabinoid system, in particular CB2 receptor antagonists, may act as novel anti-resorptive therapeutics in the treatment of human bone disease such as post-menopausal osteoporosis where excessive osteoclastic resorption contributes to the pathology of the disease.

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Conflicts of interest
None.

References


Cannabinoids and human osteoclasts


Supporting information

Additional Supporting Information may be found in the online version of this article:

**Figure S1** High throughput quantification of VNR fluorescence using a plate reader as a measure of osteoclast formation. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of M-CSF, M-CSF and RANKL or M-CSF, RANKL and 10 ng·mL⁻¹ TGFβ for 7 days. Cells were fixed and stained for the vitronectin receptor and immunofluorescence intensity measured as an indication of osteoclast number (A). Results are expressed as a percentage of M-CSF and RANKL treated cells – mean ± SEM; n = 4–8 experiments – 5 replicates each. ***P < 0.001; Student’s t-test. (B) Comparison of osteoclast number determined by counting versus plate reader VNR fluorescence. (C) Representative images of osteoclasts formed in the presence of M-CSF alone, M-CSF and RANKL or M-CSF, RANKL and TGFβ are shown with vitronectin receptor in (green) and nuclei (blue).

**Figure S2** Anandamide, 2-AG and CP 55 940 do not have a toxic effect on osteoclast precursors. M-CSF-dependent human PBMCs were cultured in the presence of RANKL and treated with anandamide (A), 2-AG (B) or CP 55 940 (C) for 7 days, after which an AlamarBlue (Invitrogen) assay was performed to assess the number of viable cells. Results are expressed as mean ± SEM (mean of 4–7 experiments with 5 replicates per experiment).

**Figure S3** Cannabinoid receptor antagonist AM251 increases human osteoclast formation. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of vh (control), 10 ng·mL⁻¹ TGFβ (positive control) or cannabinoid receptor antagonists SR141716A (141), SR144528 (144), AM251 and AM630 for 7 days and then fixed and stained for VNR. Immunofluorescence intensity was measured and expressed relative to control cultures as an indication of osteoclast number. Mean ± SEM; n = 4 experiments – 5 replicates each. ***P < 0.01, ****P < 0.001 compared with control – one way ANOVA with Bonferroni post-test.

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