Bone cell–autonomous contribution of type 2 cannabinoid receptor to breast cancer induced osteolysis

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Running title: Skeletal CB2 receptor modulates osteolysis.

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Keywords: osteolysis, cannabinoids; CB2; osteoclast; breast cancer; bone; osteoclastogenesis.

Background: CB2 is implicated in bone remodeling and tumour growth.

Results: CB2 activation enhances breast cancer induced bone cell activity and osteolysis via PI3K/AKT pathway.

Conclusion: CB2 selective antagonism have potential efficacy in cancer associated bone disease.

Significance: CB2 activation by phytocannabinoids might be detrimental in breast cancer patients with advanced malignancy.

Abstract

The cannabinoid type 2 receptor (CB2) has previously been implicated as a regulator of tumour growth, bone remodelling and bone pain. However, very little is known about the role of the skeletal CB2 receptor in the regulation of osteoblasts and osteoclasts changes associated with breast cancer. Here, we found that the CB2 selective agonists HU308 and JWH133 reduced the viability of a variety of parental and bone-tropic human and mouse breast cancer cells at high micro-molar concentrations. Under conditions in which these ligands are used at the nano-molar range, HU308 and JWH133 enhanced human and mouse breast cancer cell-induced osteoclastogenesis and exacerbated osteolysis, and these effects were attenuated in cultures obtained from CB2 deficient mice or in the presence of a CB2 receptor blocker. HU308 and JWH133 had no effects on osteoblast growth or differentiation in the presence of conditioned medium from breast cancer cells, but under these circumstances both agents enhanced PTH induced osteoblast differentiation and ability to support osteoclast formation. Mechanistic studies in osteoclast precursors and osteoblasts showed that JWH133 and HU308 induced PI3K/AKT activity in a CB2 dependent manner, and these effects were enhanced in the presence of osteolytic and osteoblastic factors such as RANKL and PTH. When combined with published work, these findings suggest that breast cancer and bone cells exhibit differential responses to treatment with CB2 ligands, depending upon cell type and concentration used. We therefore conclude that both, CB2 selective activation and antagonism have potential efficacy in cancer associated bone...
disease but further studies are warranted and ongoing.

The endocannabinoid system comprises two known receptors (CB1 and CB2), a family of endogenous ligands, and molecular machinery for ligand synthesis, transport, and inactivation (1). CB1 and CB2 receptors belong to the G protein-coupled receptor (GPCR) super-family that exhibit 44% homology at the protein level (2) and share a number of common signal transduction pathways, including adenyl cyclase (3), extracellular signal-regulated (ERK) kinases (p42/p44 MAPK) (4,5) and phosphatidylinositol 3-kinase / AKT (PI3/AKT) pathway (6). The CB1 receptors are highly expressed in the central nervous system, whereas CB2 is predominately found in the immune system and a number of other peripheral tissues (1).

Delta-9-Tetrahydrocannabinol (Δ9-THC), the main psychotropic constituent of cannabis (1), and various synthetic cannabinoid receptor ligands have been extensively investigated as potential treatments for cancer with varying results. Depending on the ligands used, cell lines studied, and disease models employed cannabinoid receptor ligands have been shown to exert both stimulatory and inhibitory effects on cancer cell proliferation and tumour progression (7-16). In general terms however, CB1 and CB2 receptor agonists have been found to have inhibitory effects on tumour cell growth, whereas antagonists and inverse agonists have been found to have stimulatory effects (7-16). Moreover, clinical studies have shown that cannabinoid receptor agonists exert analgesic and muscle relaxant properties in patients with metastatic pain (7-18). The cannabinoid-based drug Sativex®, a plant extract that contains various plant-derived cannabinoids, is approved in some countries for the treatment of cancer associated pain (reviewed in (16)), whereas the synthetic equivalents of Δ9-THC Marinol® and Cesamet® have been prescribed for the treatment of nausea and vomiting associated with cancer chemotherapy (reviewed in (16)).

Most recent interest has been focused on the potential role of CB2 selective agonists in the treatment of malignant disease, because these agents (a) lack of adverse psychotropic effects that are associated with CB1 selective ligands (19), (b) exert anti-proliferative effects on different cancer cell lines (20,21), (c) inhibit cancer-induced osteolysis and fractures (22) and (d) reduce bone pain in various preclinical models (22,23). At the present time CB2 agonists are considered to exert these effects by inhibiting tumour cell growth and by suppressing the release of cytokines and chemokines from cancer cells (24). However the cellular and molecular mechanisms by which CB2 agonists protect against tumour induced osteolysis remains incompletely understood. Over recent years we and others have reported that cannabinoid receptors and their ligands directly regulate bone cell activity and bone mass (25-32). This raises the possibility that the effects of cannabinoid receptor ligands on models of cancer-associated osteolysis might be mediated, not only by effects on tumour cells, but also by effects on bone cells. In this study we have employed genetic and pharmacological approaches to examine the mechanisms by which the skeletal CB2 pathway regulates osteolysis mediated by breast cancer.

EXPERIMENTAL PROCEDURES

Materials

The cannabinoid receptor agonists HU308, JWH133, and AM630 were purchased from Tocris Biosciences (Bristol, UK). Human MDA-MB-231 (MDA-231) and MCF7 and mouse 4T1 breast cancer cell lines were purchased from ATCC (Manassas, VA). Tissue culture medium (alpah-MEM and DMEM) were obtained from Invitrogen (Paisley, UK). The PI3 kinase inhibitor LY294002 was purchased from New England Biolabs (UK) Ltd (Hertfordshire, UK) and the inhibitor of Gi/o proteins pertussis toxin (PTX) was purchased from Sigma-Aldrich (Dorset, UK). Primers for quantitative PCR were designed using the Roche website and obtained from Invitrogen (Paisley, UK) and probes were purchased from Roche Diagnostics Ltd. (East Sussex, UK). All primary antibodies were purchased from Cell Signalling Biotechnology (MA, USA) except rabbit anti-CB2 receptor (CB2) that was purchased from Cayman Chemical (UK) and anti-actin that was obtained from Sigma-Aldrich (Dorset, UK). Mouse macrophage colony stimulating factor (M-CSF) was obtained from R&D Systems (Abingdon, UK) and receptor activator of NFkB ligand (RANKL) was a gift from Patrick Mollat (Galapagos SASU, France), and was prepared as previously described (33).

Cell culture and conditioned medium

Human MDA-231 and MCF-7 and mouse 4T1 breast cancer cells were cultured in standard DMEM (DMEM supplemented with 10% FCS, penicillin and
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For studies involving conditioned medium, breast cancer cells were cultured in standard DMEM and allowed to grow to 80% confluence over a period of 48-72 hours. Medium was removed and replaced with serum free DMEM, and then the cells were incubated for a further 24 hours. Conditioned medium from these cultures was filtered (0.22 µM filter diameter) and used fresh (10-20% v/v).

**RANKL and M-CSF mouse osteoclast culture**

Mouse osteoclast cultures were generated from bone marrow macrophages stimulated with M-CSF and RANKL as previously described (30). For studies involving conditioned medium, conditioned medium prepared as described above was added to osteoclast cultures at a concentration of (10%, v/v) in standard alpha-MEM supplemented with M-CSF (25 ng/ml) and RANKL (100 ng/ml). Cultures were then treated with vehicle or test compounds for the desired period of time. At the end of the culture period cells were fixed in 4% formaldehyde, washed with PBS and stained for Tartrate-Resistant Acid Phosphatase (TRAcP) as described in (34). TRAcP-positive cells with more than three nuclei were counted as osteoclasts.

**Bone marrow/breast cancer cell co-culture**

Bone marrow macrophages or M-CSF generated osteoclast precursors were plated into 96 well plates (10 x 10^3 cells/well) in 150 ml of standard alpha-MEM supplemented with M-CSF (25 ng/ml) and RANKL (100 ng/ml) for 6 hours prior addition of MDA-231, MCF-7 or 4T1 breast cancer cells (300 cells/well). Cultures were then treated with vehicle or test compounds for the desired period of time. At the end of the culture period cells were fixed in 4% formaldehyde, washed with PBS and stained for Tartrate-Resistant Acid Phosphatase (TRAcP). TRAcP-positive cells with more than three nuclei were counted as osteoclasts.

**Bone marrow cultures in the presence of conditioned medium**

Primary osteoblasts were isolated from the calvarial bones of 2-day-old mice by sequential collagenase digestion as previously described (35). Osteoblasts were maintained in alpha-MEM supplemented with 10% FCS and left to adhere overnight. Conditioned medium was then added to the osteoblast cultures (10% v/v) and cells were treated with vehicle or test compounds for the desired period of time. At the end of the experiment, osteoblast cultures were used for RNA isolation or to determine osteoblast number and differentiation by AlamarBlue assay and alkaline phosphatase (ALP) assay, respectively. Both assays were performed as previously described (36).

**Bone marrow cell/osteoblast co-cultures in the presence of conditioned medium**

Bone marrow cell populations containing osteoclast precursors were isolated using the Ficoll-Hypaque density gradient centrifugation technique as described in (30). These cells were then seeded in plates containing adherent primary osteoblasts in the presence of conditioned medium (10% v/v), prepared as described above. Cultures were then treated with vehicle or test compounds for the desired period of time. At the end of the culture period, cells were fixed in 4% formaldehyde, washed with PBS and stained for Tartrate-Resistant Acid Phosphatase (TRAcP). TRAcP-positive cells with more than three nuclei were counted as osteoclasts.

**Human breast cancer cell/mouse calvarial co-culture**

Neonatal mouse calvaria were isolated from 7 day-old mice, washed thoroughly in Hank’s balanced salt solution and incubated in standard alpha-MEM as described in (37). Each mouse calvaria was then divided into two halves along the median sagittal suture. Each half was placed in organ culture on a stainless steel raft in 48-well plates containing standard medium (Fig. 5A), and treated for 7 days with vehicle 0.1% DMSO or test compounds in the presence or absence of MDA-231 cells (10 x 10^3 cells/well). Osteolysis was assessed by measuring bone volume (BV) using micro computed tomography (microCT; Skyscan 1172 scanner, Skyscan, Belgium) at a resolution of 5µm. Cancer cells from MDA-231/mouse calvarial organ cultures were washed three times with ice cold PBS, lysed and supernatant was collected. Total protein (50µg) was resolved on polyacrylamide gels, transferred onto PVDF membranes (BioRAD, UK) and immunoblotted with human cleaved and total Caspase-3 antibodies (Santa
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Western Blotting

Western blot analysis was used to detect protein expression and activity in cultured cells. Cells were seeded in 12-well plates and maintained in standard medium until confluent. Prior to stimulation with test agents or vehicle, cells were incubated in serum free medium for 60 minutes. Test agents or vehicle were prepared in serum free medium and were then added for the desired period of time. Cells were then scraped, supernatant was collected and protein concentration was determined as previously described (31). Total protein (50 µg) was resolved on polyacrylamide gels, transferred onto PVDF membranes (BioRAD, UK) and immunoblotted with antibodies according to manufacturer’s instructions. Immunocomplexes with primary and secondary antibodies were visualized using a chemiluminescent detection system (Fisher Scientific) on a Syngene Genegnome bioimaging system (Fisher Scientific) (31). Levels of phosphorlated (or modified) proteins were normalized to total protein and changes were expressed as percentage of control.

Quantitative PCR

Gene expression was detected using quantitative PCR (qPCR). Total RNA was isolated and complementary DNA (cDNA) was generated as previously described (26). Primers were designed using the Ensembl genome browser and Roche website, for amplification of mouse TRAcP (forward primer: 5’-CGTCTCTGCACAGATTGCAT-3’, reverse primer 5’-AAGCGCAAACGGAATGTAAGG-3’, product size 75bp); mouse cathepsin K receptor (forward primer: 5’-CGAAAAGAGCCTCTAGCAACA-3’, reverse primer 5’-TGGTGAGCAGACGAAACTTG-3’, product size 67bp); mouse calecitomin receptor (forward primer: 5’-GGTTCTTTCCTGTCAGAAGGT-3’, reverse primer 5’-GCCCTGAAGAAGTGGTGATGTTG-3’, product size 70bp); mouse OPG (forward primer: 5’-atgaacaagtggctgtgctg-3’, reverse primer 5’-gcattctggctactgaactgca-3’); mouse RANKL (forward primer: 5’-tgaagacacactacctgactcctg-3’, reverse primer 5’-ccacaatgtgttgcagttcc-3’); and human GAPDH (forward primer: 5’-agccacatcgctcagacac-3’, reverse primer 5’-gcccaatacgaccaaatcc-3’). The PCR protocol was as follows: denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds followed by extension at 72°C for 15 seconds. Levels of gene expression were expressed as copy number per micro-gram of total RNA and GAPDH was used for normalization.

Statistical analysis

Comparison between groups was done by analysis of variance (ANOVA) followed by Dunnet’s post test using SPSS for Windows version 11. A p-value value of 0.05 or below was considered statistically significant. The half maximal inhibitory concentration (IC50) values were calculated using GraphPad Prism 4 for windows. Data are averages of three independent experiments, and reported error bars are standard deviation, unless stated otherwise.

RESULTS

Effects of CB2 receptor agonists on growth of breast cancer cell lines and bone cells

The CB2 selective agonists HU308 and JWH133 inhibited growth of parental and bone-tropic breast cancer cell lines MDA-231 and 4T1, and parental MCF7 cells in the low micro-molar range with half maximal inhibitory effects (IC50) between 5-10 µM range (Table 1). In contrast, no significant inhibitory effects on growth of osteoblasts or bone marrow derived cells were observed at concentrations of greater than 10 µM (Table 1).

CB2 receptor activation stimulates osteoblast support for osteoclastogenesis

It is known that osteoblast-like cells support osteoclastogenesis through secretion of various cytokines including macrophage colony stimulating factor (M-CSF) and receptor activator of NFκB ligand (RANKL) (38). In view of this, we examined the effects of CB2 agonists on osteoclastogenesis in bone marrow/osteoblast co-cultures exposed to conditioned medium from a variety of human and mouse breast cancer cells (Fig. 1A). Conditioned medium from human MDA-231, human MCF7 and mouse 4T1 breast cancer cells, stimulated osteoclast formation in bone marrow cell/osteoblast co-cultures compared to control cultures (Fig. 1B). Moreover, both JWH133 and HU308 at concentrations 0.1 - 1µM further enhanced the breast cancer-induced osteoclast formation in these cultures (Fig. 1B-C). This stimulatory effect of both ligands was associated an increase in the RANKL/OPG ratio (50% ± 5.5 increase with JWH133, p < 0.05; 100% ± 6.5 increase with HU308, p < 0.05) (Fig. 1D-F). However, neither of the CB2 receptor selective ligands had an effect on osteoblast proliferation (Fig. 1G). Together, these data indicates that one mechanism by which CB2 receptor...
activation in osteoblasts promotes breast cancer induced osteoclastogenesis is by increasing the RANKL/OPG ratio.

**CB2 receptor agonists enhance breast cancer-induced osteoclast formation and bone resorption**

To examine the direct effects of CB2 agonists on osteoclasts and their M-CSF generated precursors, we went on to test the effects of JWH133 and HU308 on osteoclast formation in M-CSF and RANKL stimulated bone marrow cultures in the presence or absence of conditioned medium from a variety of human and mouse breast cancer cells (Fig. 2A). Pretreatment of M-CSF generated osteoclast precursors with the CB2 receptor selective agonists JWH133 and HU308 at concentrations between 0.1 - 1µM for 1 hour prior to stimulation with RANKL (100ng/ml) enhanced the effects of M-CSF and RANKL on osteoclast formation (Fig. 2B and C), and in the same cultures further enhanced the osteoclastogenic effects of conditioned medium from human MDA-231, human MCF7 and mouse 4T1 breast cancer cells (Fig. 2B and C). In keeping with this, expression of osteoclast-specific genes, such as TRAcP, calcitonin receptor and cathepsin K, were increased by JWH133 and HU308 in these cultures (Fig. 2D). Bone resorption was also significantly enhanced by JWH133 (35% ± 14 increase) and HU308 (41% ± 1.6 increase) in similar cultures using conditioned medium from human MDA-231 (Fig. 2E). While breast cancer cell conditioned medium and the CB2-selective agonists enhanced the osteoclastogenic effects of M-CSF and RANKL, these factors alone and in combination were unable to support osteoclast formation in the absence of RANKL and M-CSF (Fig. 2F). Furthermore, addition of JWH133 and HU308 to MDA-231 conditioned medium enhanced M-CSF and RANKL induced osteoclast formation in wild type cultures, but no effect was observed in bone marrow cultures from mice lacking CB2 receptors (CB2−/− mice) (Fig. 4A-B). In accordance to this, pre-treatment with the CB2 selective antagonist/inverse agonist AM630 or the Gi/o inhibitor pertussis toxin (PTX) and the CB2 selective antagonist/inverse agonist AM630 (Fig. 4C-D). Previously, we reported that PTH, a potent stimulator of osteoblast differentiation and AKT activity (42-43), enhances alkaline phosphatase (ALP) activity in wild type calvarial osteoblasts but not in cultures from mice deficient of CB2 receptors (CB2−/− mice) (31). Here, we examined the effects of PTH on osteoblast cultures exposed to conditioned medium from MDA-231 breast cancer cells. This showed that PTH stimulated alkaline phosphatase in osteoblast cultures and that the CB2 agonists JWH133 and HU308 further increased alkaline phosphatase levels (Fig. 5A). Similarly, PTH stimulated osteoclast formation in mouse osteoblasts/bone marrow cell cultures in the presence of MDA-231 cell conditioned medium (Fig. 5B-C). Osteoclast formation was further increased by the combination of PTH and the CB2 agonists JWH133 and HU308 (Fig. 5B-C). In cultures of calvarial osteoblasts, however, JWH133 and HU308 enhanced PTH induced phosphorylation of AKT at threonine 308 (Fig. 5D-E). These data together demonstrate that breast cancer cell conditioned medium stimulates AKT phosphorylation in bone
marrow cells and that this is increased by PTH and further increased by CB2 agonists. This illustrates that another mechanism by which the CB2 pathway enhances osteoclasterosis is through AKT phosphorylation.

**Involvement of the CB2 receptor in focal osteolysis is mediated by breast cancer cells**

Next, we investigated the role of CB2 receptor activation on breast cancer-induced osteolysis in an organ culture model by using MDA-231 human breast cancer cells/mouse calvarial organ culture (Fig. 6A). Addition of MDA-231 cells to the organ cultures from wild type mice caused a dramatic decrease in calvarial bone volume, reaching 73% ± 2.9 bone loss (p < 0.01), over a 7 days culture period (Fig. 6B). Treatment with the CB2 receptor selective agonists JWH133 and HU308 increased osteolysis even further to 87% ± 2.7 (p < 0.05) and 92% ± 3.1 (p < 0.05), respectively (Fig. 6B and 6C). However, osteolysis was reduced following treatment with the CB2 selective antagonist/inverse agonist AM630. This was mirrored in a pharmacologic inactivation context, when cultures were treated with the CB2 selective antagonist/inverse agonist AM630, pointing to the fact that any form of inactivation of the CB2 receptors suppresses osteoclastogenesis in a metastatic bone environment.

Previously, we showed that alkaline phosphatase (ALP) activity, a marker of osteoblast differentiation, is enhanced by PTH in wild type calvarial osteoblasts but not in cultures from CB2-/- mice (31). Here, PTH-induced ALP activity in the presence of conditioned media was further enhanced following treatment with JWH133 and HU308, supporting the fact that activation of CB2 receptors increases osteoblast differentiation in a metastatic setting. Moreover, CB2 activation stimulates osteoblast support for osteoclastogenesis by enhancing RANKL/OPG ratio in the presence of conditioned media from breast cancer cells. It is well-established that breast cancer and bone cells secrete various factors that increase RANKL and inhibit OPG expression by osteoblasts (41). Although the signalling mechanisms by which cannabinoid receptor ligands affect bone cell differentiation and function are thus far relatively unknown, the current study suggests that in M-CSF generated osteoclast precursors and osteoblasts, cannabinoid receptor agonists enhance phosphorylation of AKT suggesting that this may be one mechanism by which CB2 activation contributes to osteoclastic and osteoblastic effects associated with osteolysis. It is worthwhile to mention that neither HU308 nor JWH133 had any significant effects on the activation and phosphorylation of a number of key signalling proteins and transcription factors down stream of cannabinoid receptor antagonists/inverse agonists.
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RANK receptor, namely IKKalpha and beta, IkB, p38, ERK1/2, MEK1/2, JNK1/2, p65NFκB, NFATc1 and cFOS (data not shown). Therefore, future studies are still needed to evaluate the mechanism by which CB2 receptor signalling in bone cells influences different aspects of AKT activity such as posttranslational modifications. Previous investigators showed that pharmacological targeting of CB2 receptors with non-psychoactive CB2-selective agonists reduced cancer-induced bone loss and bone fracture (22,24). However, these effects were attributed on the anti-proliferative actions of the CB2 selective agonists on cancer cells rather than on an inhibitory effect directed on osteoclasts specifically. Although we cannot exclude the possibility that the CB2 agonists studied here have inhibitory effects on tumour growth, the concentrations of these agents required to inhibit growth of tumour cells were three times higher than those that were shown here to enhance osteolysis that resulted from tumour conditioned medium. In addition the experiments in cultured calvarial explants and cultures from CB2 deficient mice demonstrate clearly that at least part of the effect was mediated by the cannabinoid pathway in bone cells rather than the tumour. It should also be noted that the CB2 selective agents JWH133 and HU308 appeared to exacerbate osteolysis in calvarial organ co-cultures from CB2 deficient mice. While we cannot exclude the possibility that CB2 independent effects exerted by the HU308 and JWH133 may have contributed to the effects that we observed, the present study shows that CB2 exerts bone cell-autonomous effects on differentiation of osteoclasts and exerts direct effects on osteolysis. Further studies using cultured calvarial explants and cultures from cell-specific or neuron-specific inactivation of CB2 will be required to address the relative importance of signaling by CB1 and/or other related receptors to the regulation of osteolysis described in this study.

In conclusion, our present findings suggest that inhibition of CB2 receptor signaling in the bone microenvironment may have a potential role in protecting the skeleton from the osteolysis associated with breast cancer. When combined with previous studies, these findings suggest that the skeletal CB2 receptor exhibits differential responses to treatment with CB2 ligands and raises the possibility that both CB2 selective activation and antagonism have potential efficacy in cancer associated bone disease. The potential use of CB2 receptor agonists in cases of breast cancer with bone metastases needs to be carefully explored so that any treatment regime would take into consideration and exploit both, their cell-autonomous effects in the bone microenvironment and their direct effects on tumour. For that, further in vivo studies are warranted and ongoing.
CONFLICT OF INTEREST
Dr A.I Idris and Prof. S.H Ralston are co-inventors on a patent claiming the use of cannabinoid receptor ligands as treatments for bone disease.

Patrick Mollat is an employee of Galapagos SASU (102 Avenue Gaston Roussel, 93230 Romainville, France).

AUTHOR CONTRIBUTIONS
Antonia Sophocleous – acquisition, analysis and interpretation of data, Silvia Marino – acquisition, analysis and interpretation of data, John G Logan – acquisition of data, Patrick Mollat – conception and donation of material, Stuart H. Ralston – revising article and interpretation of data, Aymen I. Idris – conception and design, acquisition, analysis and interpretation of data, writing and revising the article.
REFERENCES

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FOOTNOTES

The abbreviations used are: CB1, cannabinoid type 1 receptor; CB2, cannabinoid type 2 receptor; GPCR, G protein-coupled receptor; ERK, extracellular signal-regulated; MAPK, mitogen-activated protein kinase; Δ9-THC, delta-9-Tetrahydrocannabinol; IC50, half maximal inhibitory effects; NFkB, nuclear factor kB; RANKL, receptor activator of NFkB ligand; M-CSF, macrophage colony stimulating factor; PTX, pertussis toxin; PI3/AKT, phosphatidylinositol 3-kinase / AKT; MDA-MB-231 (MDA-231), PTH, parathyroid hormone (PTH); PBS, phosphate buffered saline; TRAcP, tartrate resistant acid phosphatase; qPCR, quantitative polymerase chain reaction; cDNA, complementary DNA; microCT, micro computed tomography; ANOVA, analysis of variance; BV, bone volume.

This work was supported in part by ECTS/AMGEN fellowship grant to Aymen Idris and a grant from the arthritis research UK (17713).
FIGURE LEGENDS

Fig. 1. The CB2 receptor selective agonists JWH133 and HU308 enhance osteoblasts support for osteoclastogenesis in a bone metastatic setting
(A) Experimental flow and time-line of the effects of conditioned medium on osteoblast differentiation and osteoblast support for osteoclastogenesis. (B) Number of osteoclasts in mouse bone marrow cell/osteoblast co-cultures in the presence or absence of the CB2 selective agonists JWH133 and HU308 (0.1 – 1 µM) following exposure to conditioned medium from human MDA-231 and MCF7 and mouse 4T1 breast cancer cells (10% v/v). The average number of TRAcP positive multi-nucleated osteoclasts with three or more nuclei in vehicle treated control cultures are as follows: osteoblast/BMC, 27±6; osteoblast/BMC/MDA-231, 46±3; osteoblast/BMC/MCF7, 38±4; osteoblast/BMC/4T1, 45±4. (C) Representative photomicrographs of multinucleated TRAcP positive osteoclasts from the experiment described in panel B. (D-F) Expression of RANKL (D) and OPG (E) as percentage of GAPDH and RANKL/OPG ratio (E) in mouse calvarial osteoblasts in the presence or absence of the CB2 selective agonists JWH133 and HU308 (1µM) following exposure to conditioned medium from human MDA-231 breast cancer cells (20% v/v). (G) Cell number in mouse calvarial osteoblasts in the presence or absence of the CB2 selective agonists JWH133 and HU308 (0.1 – 1µM) following exposure to conditioned medium from human MDA-231 breast cancer cells (10% v/v). * p < 0.05 from vehicle control; †p < 0.05 from vehicle in the presence of conditioned medium from MDA-231 breast cancer cells.

Fig. 2. The CB2 receptor selective agonists JWH133 and HU308 enhance breast cancer-induced osteoclast formation and bone resorption
(A) Experimental flow and time-line of the effects of CB2 receptor selective agonists on conditioned medium-induced osteoclast formation, survival and activity. (B) Number of osteoclasts in bone marrow cultures stimulated with M-CSF (25ng/ml) and RANKL (100ng/ml) after exposure to conditioned media from human MDA-231 and MCF7 and mouse 4T1 breast cancer cells (10% v/v) in the presence or absence of the CB2 selective agonists JWH133 and HU308 (0.1 – 1µM). The average number of TRAcP positive multi-nucleated osteoclasts with three or more nuclei in vehicle treated control cultures are as follows: BMC, 25±2; BMC/MDA-231, 45±9; BMC/MCF7, 49±7; BMC/4T1, 61±3. (C) Representative photomicrographs of multinucleated TRAcP positive osteoclasts from the experiment described in panel B. (D) Expression of the osteoclast specific genes TRAcP, calcitonin receptor and cathepsin K in bone marrow cultures stimulated with M-CSF (25ng/ml) and RANKL (100ng/ml) after exposure to conditioned medium from human MDA-231 breast cancer cells (20% v/v) in the presence or absence of the CB2 receptor selective agonists JWH133 and HU308 (1µM). (E) Resorbed area bone marrow cultures stimulated with M-CSF (25ng/ml) and RANKL (100ng/ml) after exposure to conditioned medium from human MDA-231 breast cancer cells (20% v/v) in the presence or absence of the CB2 selective agonists JWH133 and HU308 (1µM). (F) Number of mature osteoclasts after exposure to conditioned medium from human MDA-231 breast cancer cells (10% v/v) in the presence or absence of the CB2 selective agonists JWH133 and HU308 (0.1 – 1µM) following withdrawal of RANKL (100ng/ml) or M-CSF (25ng/ml). * p < 0.05 from vehicle control; †p < 0.05 from vehicle in the presence of conditioned medium from MDA-231 breast cancer cells.

Fig. 3. JWH133 and HU308 induce PI3K/AKT activation in osteoclasts in the presence and absence of RANKL
(A) Western blot analysis of AKT phosphorylation in M-CSF generated bone marrow cells exposed to the CB2 selective agonists JWH133 or HU308 (1µM) for 15 min. (B) Quantification of AKT phosphorylation from the experiment described in panel A. (C) Western blot analysis of AKT phosphorylation in M-CSF generated bone marrow cells exposed to the CB2 selective agonists JWH133 or HU308 (1µM) for 24 hours. (D) Quantification of AKT phosphorylation from the experiment described in panel C. (E) Western blot analysis of AKT phosphorylation in bone marrow cultures stimulated with M-CSF after exposure to RANKL in the presence or absence of the CB2 selective agonists JWH133 and HU308 (1µM). (F) Quantification of AKT phosphorylation from the experiment described in panel E.
Fig. 4. JWH133 and HU308 enhance breast cancer-induced osteoclast formation via CB2 receptors in a PTX sensitive manner and a PI3K/AKT mechanism
(A) Number of osteoclasts in bone marrow cultures stimulated with mouse M-CSF and RANKL from wild type and CB2 deficient mice (CB2−/−) following treatment with the CB2 selective agonists JWH133 and HU308 (1 µM) and exposure to conditioned medium from human MDA-231 breast cancer cells (10% v/v), in the presence or absence of the CB2 selective antagonist/inverse agonist AM630 (3 µM) or the Gi/o inhibitor pertussis toxin (PTX, 10 µM). The average number of TRAcP positive multinucleated osteoclasts with three or more nuclei in wild type (WT) control cultures is 35±6. (B) Representative photomicrographs of multinucleated TRAcP positive osteoclasts from the experiment described in panel A. (C) Western blot analysis of AKT phosphorylation in bone marrow cultures stimulated with M-CSF and RANKL, exposed to conditioned medium from MDA-231 breast cancer cells (20% v/v) and then treated with the CB2 selective agonists JWH133 and HU308 (1 µM) in the presence or absence of the CB2 selective antagonist/inverse agonist AM630 (1 µM), PI3K/AKT inhibitor LY294002 (10 µM) or pertussis toxin (PTX, 10 µM). (D) Quantification of phosphorylated AKT at serine 473 as percentage of total AKT from the experiment described in panel C. *p < 0.05 from vehicle control, +p < 0.05 from WT vehicle in the presence of conditioned medium from MDA-231 breast cancer cells; $p < 0.05 from AM630, LY294002 or PTH treated. CM, conditioned medium; kDa, kilo dalton; M, marker; PTX, pertussis toxin.

Fig. 5. The CB2 receptor selective agonists JWH133 and HU308 enhance PTH induced osteoblasts and osteoclast changes in a bone metastatic setting.
(A) Alkaline phosphatase activity in mouse calvarial osteoblasts pre-exposed to conditioned medium from human MDA-231 breast cancer cells (10% v/v) then treated with the CB2 selective agonists JWH133 and HU308 (1 µM) in the presence of PTH (100 ng/ml). (B) Number of osteoclasts in mouse osteoblasts/bone marrow cell cultures pre-exposed to conditioned medium from human MDA-231 breast cancer cells (10% v/v) and then treated with the CB2 selective agonists JWH133 and HU308 (1 µM) in the presence of PTH (100 ng/ml). The average number of TRAcP positive multi-nucleated osteoclasts with three or more nuclei in wild type (WT) control cultures is 43±7. (C) Representative photomicrographs of multinucleated TRAcP positive osteoclasts from the experiment described in panel B. (D) Western blot analysis of AKT phosphorylation in mouse calvarial osteoblast cultures pre-exposed to conditioned medium from human MDA-231 breast cancer cells (20% v/v) and then treated with the CB2 selective agonists JWH133 and HU308 (1 µM) in the presence of PTH (100 ng/ml). (E) Quantification of phosphorylated AKT at threonine 308 as percentage of total AKT from the experiment described in panel D. *p < 0.05 from vehicle control; +p < 0.05 from PTH in the presence of conditioned medium from MDA-231 breast cancer cells. CM, conditioned medium; kDa and kilo dalton; M, marker.

Fig. 6. JWH133 and HU308 exacerbate breast cancer-induced osteolysis via CB2 receptor activation.
(A) Graphic representation of human breast cancer cell/mouse calvaria organ co-culture system. (B) Total bone volume loss in mouse calvaria bone from wild type and CB2 deficient mice (CB2−/−) co-cultured with human MDA-231 breast cancer cells (10 x 10^5 cells/well) in the presence or absence of the CB2 selective agonists JWH133 and HU308 (1 µM) or the CB2 selective antagonist/inverse agonist AM630 (1 µM). (C) Representative photomicrographs of microCT scans and TRAcP and Aniline stained histological sections of mouse calvarial bone from the experiment described in panel B, showing osteolysis. (D) Cell number of human MDA-231 cells in breast cancer cell/mouse calvaria organ co-culture system treated with the CB2 selective agonists JWH133 and HU308 (1 µM) for 7 days. (E) Western blot analysis of total and cleaved Caspase3 as well as actin in human MDA-231 cells in breast cancer cell/mouse calvaria organ co-culture system treated with the CB2 selective agonists JWH133 and HU308 (1 µM) for 7 days. *p < 0.05 from vehicle control; +p < 0.05 from vehicle in the...
presence of MDA-231 breast cancer cells; $^5 p < 0.05$ from wild type (WT) cultures treated with JWH133 and HU308 alone in the presence of MDA-231 breast cancer cells. kDA and kilo dalton; M, marker.

**TABLES**

**Table 1. Effects of CB2 receptor selective agonists (half-maximal inhibitory concentration, IC50) on growth of breast cancer and bone cells in vitro.** Cell numbers were measured after 48 hours of continuous exposure to test agents. Cell viability was measured after 48 hours of continuous exposure. Viability assay and calculation of half maximal inhibitory concentrations (IC50) have been performed as described under “Materials and Methods”. Values are expressed as means ± sd and are obtained from 5 independent experiments. BT denotes bone tropic and pre-osteoclasts refers to M-CSF generated osteoclast precursors.

<table>
<thead>
<tr>
<th></th>
<th>JWH133 (IC50, µM)</th>
<th>HU308 (IC50, µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-231</td>
<td>9.87 ± 1.1</td>
<td>9.3 ± 1.5</td>
</tr>
<tr>
<td>MDA-231-BT</td>
<td>7.24 ± 2.1</td>
<td>6.11 ± 1.6</td>
</tr>
<tr>
<td>MCF7</td>
<td>8.71 ± 0.9</td>
<td>5.83 ± 1.1</td>
</tr>
<tr>
<td>4T1</td>
<td>8.87 ± 1.6</td>
<td>8.30 ± 1.7</td>
</tr>
<tr>
<td>4T1-BT</td>
<td>6.33 ± 0.8</td>
<td>5.51 ± 2.1</td>
</tr>
<tr>
<td>Pre-Osteoclasts</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
</tr>
<tr>
<td>Osteoblasts</td>
<td>&gt; 10</td>
<td>&gt; 10</td>
</tr>
</tbody>
</table>
Skeletal CB2 receptor modulates osteolysis

FIGURES

Figure 1

A

B

C

D

E

F

G
Figure 2

A

B

C

D

E

F

Skeletal CB2 receptor modulates osteolysis.
Skeletal CB2 receptor modulates osteolysis

Figure 3

A

B

C

D

E

F

Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308

Akt
pAkt-473
pAkt-308
Akt
pAkt-473
pAkt-308
Akt
pAkt-473
pAkt-308
Akt
pAkt-473

RANKL

Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308
Vehicle
JWH133
HU308

Akt phosphorylation (% total Akt)
Figure 5

A

Osteoblast differentiation (ALP level) in viable cells

B

Osteoclast number (% control)

C

Control vehicle MDA-231-CM PTH vehicle

MDA-231-CM PTH JWH133

MDA-231-CM PTH HU308

D

Akt pAkt-308

E

Patched expression (% total Akt expression)

vehicle JWH133 HU308

0 1.0 (μM)

MDA-231-CM

Skeletal CB2 receptor modulates osteolysis
Figure 6

A. Female 2-day old mouse

B. Osteolytic bone loss (% control)

C. WT and CB2-/-

D. MDA-231 viability (% control)

E. Actin and C3
Bone cell-autonomous contribution of type 2 cannabinoid receptor to breast cancer-induced osteolysis
Antonia Sophocleous, Silvia Marino, John G. Logan, Patrick Mollat, Stuart H. Ralston and Aymen I. Idris

J. Biol. Chem. published online July 20, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M115.649608

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