Article:
Marino, S. and Idris, A. orcid.org/0000-0003-4327-5306 (2017) Emerging therapeutic targets in cancer induced bone disease: A focus on the peripheral type 2 cannabinoid receptor. Pharmacological Research, 119. pp. 391-403. ISSN 1043-6618

https://doi.org/10.1016/j.phrs.2017.02.023

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PII: S1043-6618(16)31380-9
DOI: http://dx.doi.org/doi:10.1016/j.phrs.2017.02.023
Reference: YPHRS 3518

To appear in: *Pharmacological Research*

Received date: 18-12-2016
Revised date: 26-1-2017
Accepted date: 27-2-2017

Please cite this article as: Marino S, Idris AI, Emerging therapeutic targets in cancer induced bone disease: a focus on the peripheral type 2 cannabinoid receptor, *Pharmacological Research* (2017), http://dx.doi.org/10.1016/j.phrs.2017.02.023

TITLE:

Emerging therapeutic targets in cancer induced bone disease: a focus on the peripheral type 2 cannabinoid receptor

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CONFLICT OF INTEREST

Dr Aymen I. Idris is an inventor on a patent concerning the use of cannabinoid receptor ligands as treatments other bone diseases. Dr Silvia Marino reports that she has no conflict of interest.

Summary

Skeletal complications are a common cause of morbidity in patients with primary bone cancer and bone metastases. The type 2 cannabinoid (Cnr2) receptor is implicated in cancer, bone metabolism and pain perception. Emerging data have uncovered the role of Cnr2 in the regulation of tumour – bone cell interactions and suggest that agents that target Cnr2 in the skeleton have potential efficacy in the reduction of skeletal complications associated with cancer. This review aims to provide an overview of findings relating to the role of Cnr2 receptor in the regulation of skeletal tumour growth, osteolysis and bone pain, and highlights the many unanswered questions and unmet needs. This review argues that development and testing of peripherally-acting, tumour-, Cnr2-selective ligands in preclinical models of metastatic cancer will pave the way for future research that will advance our knowledge about the basic mechanism(s) by which the endocannabinoid system regulate cancer metastasis, stimulate the development of a safer cannabis-based therapy for the treatment of cancer and provide policy makers with powerful tools to assess the science and therapeutic potential of cannabinoid-based therapy. Thus, offering the prospect of identifying selective Cnr2 ligands, as novel, alternative to cannabis herbal extracts for the treatment of advanced cancer patients.

Preparations of Cannabis Sativa L. plants have been used for medicinal and recreational purposes for thousands of years and its constituents are known to modulate a diverse set of physiological responses through interaction with the endogenous cannabinoid (endocannabinoid) system [1,2]. The endocannabinoid system consists of a family of receptors, endogenous ligands and the molecular machinery for their synthesis, transport and metabolism (reviewed in [3]). The majority of biological effects associated with cannabinoid receptors are mediated by endocannabinoid ligands, plant-derived (phytocannabinoids), and various synthetic compounds through their interactions with the classic cannabinoid type 1 (Cnr1) and type 2 (Cnr2) receptors albeit with different degrees of selectivity [4,5]. Physiological processes associated with the activation of the endocannabinoid system include neurotransmission, pain perception, memory and learning, emotions, appetite, motor and endocrine functions, cardiovascular homeostasis and immune response (reviewed in [6-10]). There is increasing evidence that most members of the endocannabinoid system of ligands, receptors and enzymes exert significant effects on tumour cell growth, motility, invasion, spread and colonisation of distant organs [6,11,12]. Of relevance to this review is the Cnr2 receptor has been detected in bone and cancer cells, and its pharmacological and genetic modulation have been shown to influence bone cell activity and bone remodelling in health and in disease. Thus, this article focuses on the role of Cnr2 in skeletal tumour growth, osteolytic bone damage and bone pain and argues in favour of the notion that therapeutic targeting of the peripheral Cnr2 may be of value for the reduction of skeletal complications associated with various metastatic cancers.

1. The peripheral Cnr2 receptor

The CNR2 gene, which encodes the Cnr2 receptor, is located on chromosome 1p36 in human [13]. Structurally, Cnr2 shares 44% and 68% amino acid and transmembrane region homology with Cnr1 receptor, but these two classic cannabinoid receptors are functionally not identical as demonstrated by the different binding specificity of cannabinoid agonists and antagonists to either receptor [14]. Cnr2 is differentially expressed in highly localised regions of the central

nervous system and peripheral tissues (Figure 1) and its activation is associated with various physiological effects in a discrete and tissue specific manner [15].

1.1 Cnr2 expression and function

The Cnr2 receptor is widely expressed in a number of peripheral tissues including liver, colon, pancreas, kidney, myocardium, testis, ovarium, uterus and endothelial cells of various origin (Figure 1) where it is implicated in a diverse range of physiological and pathological processes (reviewed in [16]). In the immune system, the Cnr2 receptor is highly expressed in T and B lymphocytes, monocyte/macrophages, dendritic cells, natural killer cells and neutrophils and a number of studies have demonstrated that Cnr2 in these cells is responsible for cannabinoid-mediated anti-inflammatory and immune-modulating effects [17-20]. Expression of functional Cnr2 in peripheral and sensory neurons, particularly nociceptive neurons, confirms the role this receptor in the regulation of inflammatory, neuropathic and cancer-related pain [21-23]. In the skeleton, Cnr2 is detected in various cells that found within the bone marrow cavity including monocytes and macrophages (pre-osteoclasts), in cells that reside on the bone matrix such as human and murine osteoclasts (bone resorbing cells), osteoblasts (bone forming cells) and their precursors, and in osteocytes embedded within bone matrix [24-29]. The expression levels of Cnr2 in osteoblasts, osteoclasts, and osteocytes are significantly higher than the reported for Cnr1 [24,27-29]. Furthermore, unlike the Cnr1 receptor, Cnr2 has not been detected in the neuronal fibres intervening the skeleton [28,30-32].

1.2 Cnr2 ligands

A plethora of endocannabinoids, phytocannabinoids and synthetic ligands bind to Cnr2 receptor (Table 1) [33,34]. The endocannabinoid ligand 2-arachidonoylglycerol (2-AG) and N-arachidonoylethanolamine (anandamide, AEA) are polyunsaturated fatty acids derived from hydrolysis of membrane phospholipids on demand (reviewed in [35,36]). Both endocannabinoids serve as a precursor to free fatty acids including arachidonic acid and act as lipid signalling molecules in a site- and in a time-specific manner (reviewed in [37-39]). The action of 2-AG is terminated by enzymatic hydrolysis mediated primarily by the monoacylglycerol lipase (MAGL), whereas AEA is mainly metabolised by serine hydrolases

fatty acid amide hydrolase (FAAH) [6,11,38,40,41]. Both endocannabinoids bind with high and similar affinity to Cnr1 receptor. 2AG act as a full agonist with higher efficacy for Cnr2 receptor whereas anandamide has been classified as a partial agonist (Table 1) [42-45]. Cnr2 is also a target to a number of the phytocannabinoids including psychotropic ∆9-tetrahydrocannabinol (∆9-THC) and non-psychoactive cannabidiol (CBD) (Table 1). The pharmacology of these ligands is complex, incompletely understood; a number of studies have reported that most phytocannabinoids bind Cnr2 as partial agonist or antagonist depending on the ligand and receptor expression levels in the tissue (reviewed in [33,46,47]). Several synthetic cannabinoids, mostly structurally similar to phytocannabinoids, such as JWH133, JWH015, HU308 [48] and AM1241 act as Cnr2 agonist and/or inverse agonist depending on the tissue and species [49] (Table 1). Conversely, Cnr2 selective agents such as AM630 and SR144528 act as either silent antagonists or as inverse agonists by activating downstream pathways in an opposite fashion from Cnr2 agonists [47]. Other synthetic cannabinoid receptor ligands including WIN55-212-2 have been reported to equally activate both Cnr2 and Cnr1 [43]

1.3 Cnr2 structure and signalling

The Cnr2 receptor is a single peptide seven-transmembrane domain receptor that belongs to the family of G protein-coupled receptors (GPR). Cnr2 contains an extracellular glycosilated N-terminus and an internal C-terminus domain that is coupled to a Gi/o protein (Figure 2) [17,50,51]. Cnr2 activation negatively regulates adenylyl cyclase activity [52], causing a reduction of intracellular level of cyclic adenosine monophosphate [53,54], that in turn leads to the modulation of an array of signalling pathways (Figure 2). Numerous studies have shown that Cnr2 selective ligands regulate cell proliferation, differentiation, transformation and death by triggering the activation of three major components of the mitogen-activated protein kinase (MAPK), namely extracellular signal-regulated kinases1/2 (ERK1/2), p38 and c-Jun N-terminal kinases (JNK). Cnr2 was also found to exert apoptosis, necrosis and autophagy through the modulations of the Akt-phosphoinositide 3´-kinase (PI3K), AMP-activated kinase (AMPK) and ceramide synthesis. Furthermore, Cnr2 influences cell motility in particular migration and invasion by regulating the levels of intracellular calcium, and the expression and activity of

adhesion molecules like ICAM or VCAM, matrix metalloproteinases, focal adhesion kinase (FAK) and small GTP binding proteins RhoA (Figure 2). Cnr2 activation is also associated with inhibition of nuclear factor of kappa B (NFκB) and cyclooxygenase-2 (COX-2) that often leads to significant reductions of levels of various pro-inflammatory mediators (reviewed in [53,55-59]).

2. Regulation of bone metabolism by Cnr2

The skeleton is a dynamic and cellulary diverse tissue that is constantly repaired by a complex process termed the bone remodelling cycle (Figure 3) (reviewed in [60]). The main cellular components of the bone remodelling cycle are osteoblasts (bone forming cells), osteoclasts (bone resorbing cells), lining cells and osteocytes, and the action of these cells together play an important role in the regulation of bone growth, remodelling, loss and regeneration [61]. The action of bone cells is coordinated by mechanical factors and various local and systemic mediators including hormones, cytokines and neural peptides [62,63]. The first phase of the bone remodelling cycle is bone resorption, during which old bone tissue is removed by the multinucleated, highly motile and specialised osteoclasts (Figure 3) [61]. Expansion of the osteoclast progenitor pool from hematopoietic cells and the survival and function of mature osteoclasts are primarily driven by the osteoblastic-derived cytokines, macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor kappa-B ligand (RANKL) [64-66]. Following bone resorption, mononuclear macrophage-like cells remove the remaining organic residues that in turn initiate the formation of the cement line which marks the limit of bone resorption and joins the new bone to old bone (Figure 3) [61,67,68]. Bone formation is triggered by mature osteoclast apoptosis and osteoblasts depositing new bone and matrix proteins in the newly resorbed site (Figure 3) [60]. Adipocytes are one of the most abundant cell types found in the bone marrow of long bones and a number of studies have shown that their differentiation is enhanced in aged skeleton at the expense of osteoblasts [69,70]. In human, the processes of bone resorption and bone formation last approximately ten days and three months, respectively [71]. The bone formation phase ends with 65% of osteoblasts undergoing apoptosis and the remaining osteoblasts are either buried within the newly deposited matrix as

osteocytes or converted to lining cells that cover the majority of quiescent bone surface [61]. The newly remodelled bone module enters into a resting phase, where quiescent lining cells await activation in the next bone remodelling cycle [68].

2.1 Cnr2 is expressed in the skeleton

The classical cannabinoid receptors Cnr1 and Cnr2 and their related GPR55 and vanilloid receptors are expressed in skeletal tissues. A number of investigators have demonstrated that Cnr2 is highly expressed in murine and human bone cells compared to Cnr1 [24,72,73]. The expression of Cnr2, at both mRNA and protein levels, was detected in various cells of bone marrow origins including M-CSF generated macrophages (pre-osteoclasts), RANKL-stimulated multinucleated osteoclasts [27-29] and bone marrow-derived stromal cells [24]. Mature calvarial osteoblasts and osteoblast-like cell lines also express Cnr2 [24,73] and possess the machinery for the synthesis and metabolism of the Cnr2-selective endocannabinoid 2AG [29,74]. Importantly, level of expression of Cnr2 in experimental animals and human has been found to be associated with bone growth, bone remodelling and age-related bone loss [24,75]. Unpublished work by our group have recently found that Cnr2 in mature calvarial osteoblasts is expressed in two different forms most likely representing a glycosylated and a non-glycosylated form of Cnr2. Bone marrow and calvarial osteoblasts mainly expressed the non-glycosylated form of Cnr2 whereas macrophages and osteoclasts mainly expressed the glycosylated Cnr2 form (Sophocleous, PhD thesis, University of Edinburgh Research Archive).

2.2 Protection against age-related bone loss by Cnr2

The Cnr2 receptor plays an important role in bone cell activity and bone loss during aging. Ofek and colleagues were the first workers to show that aged mice with deficiency in Cnr2 on a C57BL/6 genetic background had accelerated age-related trabecular bone loss and cortical expansion when compared to wild type littermates [24]. Consistent with these findings, we have observed that Cnr2 deficient mice of similar age exhibited accelerated osteoporosis characterised by uncoupling of bone resorption from bone formation [73]. This suggests that activation of Cnr2 enhances osteoblast and osteoclast activity but causes bone loss due to the increase in osteoblast differentiation and bone formation rate in aged animals does not

compensate for the excessive bone resorption. Histomorphometric analysis of bones and *in vitro* cultures have confirmed these findings and showed that bone marrow stromal cells and calvarial osteoblasts isolated from Cnr2 deficient mice produce less osteoclasts and form less bone nodules, indicative of a defect in both osteoclast and osteoblast differentiation [25,73]. Moreover, activation of Cnr2 receptors in osteoblasts by endocannabinoids and synthetic agonists resulted in a significant increase in the levels of receptor activator of NFκB ligand (RANKL) and osteoprotegerin (OPG) production, implicating Cnr2 in the regulation of osteoblast-osteoclast crosstalk [76]. Consistently, pharmacological activation of Cnr2 receptor signalling using the Cnr2-selective non-psychotropic agonist HU308 protected against bone loss induced by oestrogen deficiency by stimulating bone formation rather than inhibiting osteoclast number and bone resorption [73]. Collectively, these findings demonstrate that the Cnr2 receptor plays a role in bone cell activity and bone metabolism in rodents, which has led to the notion that selective therapeutic targeting of the skeletal Cnr2 receptor may be of value for the prevention of excessive bone damage associated with a variety of bone diseases.

2.3 Regulation of peak bone mass by Cnr2 receptor

We have recently reported that adult mice deficient in Cnr2 were partially protected from ovariectomy induced bone loss compared to wild type littermates [73] and Cnr2 knockout mice on CD1 background had increased trabecular peak bone mass [77]. These findings are contrary to studies in ageing mice but are consistent with pharmacological studies that have shown that selective blockade of Cnr2 using the antagonist/inverse agonist AM630 prevented ovariectomy induced bone loss [25]. Detailed *in vitro* and *in vivo* analysis of bone cell differentiation and activity in adult Cnr2 knockout mice and wild type littermates after treatment with the Cnr2 inverse agonist AM630 showed that these effects were likely due to a reduction in osteoclast number and activity rather than an increase in bone formation. Altogether, these results suggest that pharmacological and genetic modulation of Cnr2 affects bone mass differentially in young and aged skeleton, while osteoclast inhibition associated with Cnr2 deficiency in adult mice results in high peak bone mass and prevents bone loss associated with oestrogen deficiency. Contrary to this, we did not detect any abnormalities in peak bone mass

in young Cnr2 knockout mice in C57Bl'6 background [78] whereas Ofek and colleagues have observed a small reduction in bone mass in the same background and at similar age [24,77]. Recent work by our group have attributed these differences in skeletal phenotypes that we and others have observed to inherent genetic differences between strains and/or a consequence of Cnr2 deficiency affecting the expression of large numbers of genes in different strains of mice [77,79].

3. Role of Cnr2 in cancer

Metastasis to distant sites is a common cause of morbidity and death in advanced cancer patients. Although advances in early detection, chemotherapy and radiotherapy have significantly reduced death and disease recurrence, acquired resistance to anti-cancer therapy and cancer-induced pain still remain major clinical problems. Thus, treatments aimed at blocking cancer metastasis and attenuating pain would prove to be beneficial in terms of clinical outcomes in cancer patients with advanced disease. All members of the endocannabinoid system of ligands, receptors and enzymes have been implicated in tumour growth, metastasis and pain. However, the use of cannabis-based therapies in cancer patients has been limited and restricted to palliative use [56,80]. In recent years, interest in the peripheral Cnr2 as an attractive, druggable target for the treatment of primary cancer, metastases and pain has been growing within the cancer research community (reviewed in [23,57,81,82]). The use of small-molecule agonists and antagonists to investigate the effects of Cnr2 manipulation on tumour growth and pain perception in preclinical mouse models of cancer revealed conflicting results. Whilst Cnr2 is highly expressed in various tumours and its expression was shown to correlate with poor prognosis [83,84], both pro- and anti-tumour effects have been associated with its activation. Furthermore, the mechanisms by which Cnr2 exerts these effects vary between tumour cell types, animal models and cancers [56,83-90]. This review highlights the role of Cnr2 in skeletal tumour growth, bone remodelling and pain perception, and focuses on the effects of various Cnr2 selective ligands that have been reported to alleviate skeletal complications associated with cancer.

3.1 Role of Cnr2 in cancer induced bone disease

Cancer induced bone disease (CIBD) is common in patients with primary bone cancer, bone metastases and those who are receiving chemotherapy [91-95]. Excluding haematological malignancies, malignant primary bone tumours such as sarcomas are rare and represent less than 0.2% of all cancers in the developed world [96], whereas secondary bone metastases are relatively more common and constitute more than 99% of bone tumours [97]. Solid tumours of breast and prostate origin that develop metastatic potential have a remarkably high rate of metastasis to bone, with an incidence of 80% and 70% respectively (reviewed in [91,92,98]). In contrast, only 30% of patients with lung, colon, stomach, bladder, uterus, rectum, thyroid, or kidney carcinoma develop cancer associated bone disease [99]. Clinical complications associated with CIBD in advanced cancer patients are life debilitating and include severe bone pain, restricted mobility, pathological fracture, hypercalcemia and nerve compression [91,100]. CIBD is virtually incurable and life expectancy for patients with the condition is low, variable and depends on the primary tumour type, site and number of bone metastases [101,102].

CIBD is mainly caused by abnormal and uncontrolled growth of cancer cells in bone that progressively alters bone cell activity and turnover and ultimately lead to excessive bone loss (Figure 3). Osteotropic cancer cells, whether of bone origin or metastatic, are incapable of causing bone resorption but these cells secret various osteolytic and osteoblastic factors thereby causing bone lesions [95,103]. Tumour-derived factors such us parathyroid hormone-related peptide (PTHrP), transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2), bone morphogenetic protein (BMPs), platelet derived growth factors (PDGF), endothelin-1 (ET-1), insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF) and interleukins directly or indirectly disrupt the normal balance between osteoblasts and osteoclasts, increasing osteoblast differentiation and activity, resulting in excessive osteoclast formation and bone resorption (reviewed in [104-106]). Cancer cells in bone (i.e. osteotropic cells) are also capable of stimulating osteoclast activity directly or through their ability to instruct immune cells or osteoblasts to release osteolytic factors such as IL-11, IL-6, IL-8, IL-17, PTHrP, tumour necrosis factor –α (TNF- α), VEGF, matrix metalloproteinases (MMPs),

and RANKL (reviewed in [105,107-110]). Bone resorption by osteoclasts releases factors from the bone matrix such as TGF-β and IGF-1 that further enhance tumour growth and bone destruction [111,112]. Bone metastases are classified according to the radiological appearance as osteolytic, caused by an excessive osteoclastic bone resorption or osteoblastic, characterized by an excessive and uncontrolled bone formation [105,107,113]. Current bone targeted therapies strongly inhibit osteoclast formation and bone resorption but they have no significant, direct impact in skeletal tumour burden and CIBD-related bone pain [99,100,114]. This necessitates the need to identify additional therapeutic strategies that - alone or in combination with conventional anti-cancer therapies - reduce tumour skeletal burden, prevent bone metastases, halt recurrence, and attenuate pain in advanced cancer patients with CIBD.

The endocannabinoid system is implicated in pain perception and tumour growth, and pharmacological and genetic manipulation of Cnr2 has been found to reduce the progression of CIBD. To date, three distinct but not exclusive mechanisms have been described for Cnr2-mediated anti-cancer and osteo-protection. First, agents that activate and block Cnr2 directly act on cancer cells and induce apoptotic and necrotic cell death. Secondly, by inhibiting osteoclast formation and bone resorption, Cnr2 selective ligands reduce osteolysis and bone damage. Thirdly, activation of Cnr2 has been found to attenuate cancer-induced bone pain by (a) directly targeting various pain related pathways and/or (b) indirectly inhibiting bone damage.

3.1.1 Regulation of skeletal tumour cell growth by Cnr2

A large body of evidence indicates that modulation of Cnr2 receptor impairs the progression and growth of various tumours and cell lines such as breast, prostate and lung cancer cells which are all known to metastasize to bone [81,82,85]. Although the mechanisms downstream of Cnr2 responsible for these effects are not completely understood, it is evident that Cnr2 selective ligands inhibit cancer cell proliferation [115-119] and induce apoptosis-mediated cell death *in vitro* and *in vivo* [89,90,120]. Various investigators have observed a significant reduction of cancer cell invasion and metastases following treatment to Cnr2

selective ligands [121-123]. These effects were mainly attributed to inhibition of the proteolytic matrix metalloproteinases MMP2 and MMP9, and the increase in the expression of their inhibitor TIMP-1 [83,123-126]. Activation of Cnr2 receptor in tumour cells was also found to be associated with significant inhibition of tumour angiogenesis due to the reduction of tumour-derived vascular endothelial growth factor (VEGF) [118,127]. Autocrine inhibition of VEGF and other tumour-derived mediators such as angiopoietin 2 by Cnr2 activation reduces tumour cell motility and the support of vascular endothelial cells present in the tumour microenvironment to tumour cells [128]. Altogether, these findings indicate that Cnr2 plays a significant role in tumour cell growth and motility.

The fact that Cnr2 is highly expressed in bone cells compared to Cnr1 has led a number of investigators to pursue Cnr2 as a candidate therapeutic target for the treatment of CIBD. One of the key pathological features of CIBD is skeletal tumour burden due to excessive proliferation and increased survival of tumour cells in bone [129]. However, thus far the evidence that targeting the Cnr2 receptor could be of value in the reduction of skeletal tumour growth is confined to limited *in vitro* and preclinical studies carried out in mouse models of breast cancer- and osteosarcoma-induced CIBD. Lozano-Ondoua and colleagues were the first investigators to report that pharmacological modulation of Cnr2, using Cnr2 selective agonist JWH015, reduced the number of the breast cancer cell line 66.1 in the intramedullary cavity of mice after intra-femoral injection [130]. Further *in vitro* studies using the same cell line confirmed the anti-proliferative effects of this compound and showed that JWH015 and AM1241 – which have been reported to act as Cnr2 agonist and inverse agonist [49,131,132] – induced 66.1 cell apoptosis at microMolar concentrations. In broad agreement with these findings, we have recently observed that the Cnr2 selective agonists JWH133 and HU308 reduced the proliferation of naive and osteotropic mouse 4T1 and human MDA-MB-231 breast cancer cells in a concentration dependent manner with an IC50 at a microMolar range [76]. Interestingly, both agents were more active and exerted more anti-proliferative effects on the osteotropic mouse 4T1 and human MDA-MB-231 cells when compared to their naïve, parental clones [76]. This suggests that osteotropic cancer cells are more sensitive to Cnr2

activation but further work is needed. Work performed by Hsu *et al*. reported that the endocannabinoid and non-selective Cnr1/2 agonist anandamide induced osteosarcoma cell death via a mechanism mediated by p38 MAPK phosphorylation and caspase-3 activation, consistent with apoptosis-mediated cell death [133]. Further studies in osteosarcoma by Notaro *et al*. showed that the synthetic and non-selective Cnr1/2 agonist WIN55,212-2 induced morphological changes in osteosarcoma cells *in vitro* consistent with autophagy-mediated cell death but failed to induce apoptotic death under these conditions [134]. This finding was not surprising because it was previously reported that exposure to cannabinoid ligands induces autophagy in other cancer cell lines [90,135-137]. The authors of this study also demonstrated that WIN55,212-2 sensitized osteosarcoma cells to apoptosis and significantly enhanced the antitumor activity of TRAIL [134] and Adriamycin [138]. In addition, emerging evidence suggests that targeting Cnr2 could be of value in the prevention of tumour cells homing to bone. Nasser *et al*. reported that activation of Cnr2 reduces breast cancer progression and metastasis via a mechanism mediated by inhibition of chemokine ligand 12 (CXCL12) [139], one of the key factors implicated in the progression of bone metastasis (reviewed in [140-142]). However, the authors of this article have not directly investigated the effects of pharmacological modulation of Cnr2 in models of bone metastases. GPR55 receptors which were found to be overexpressed in a wide variety of cancer cell lines and human malignant tumors [143], are implicated in the modulation of cancer cell fate [82,144,145]. Bearing in mind that both Cnr2 and GPR55 are highly expressed in the skeleton [25,73,146], it is tempting to speculate that Cnr2-GPR55 heterodimers [147,148] may also play a role in tumour growth in bone and agents that target both receptors may be of value in the reduction of skeletal tumour burden.

3.1.2 Regulation of osteolysis by Cnr2

The major clinical complication associated with CIBD in advanced cancer patients is osteolytic bone destruction associated with enhanced osteoclast formation, survival and function. Tumour cells are known to release a variety of factors that directly stimulate osteoclastogenesis and indirectly enhance the ability of osteoblasts and immune cells such

as T lymphocytes and dendritic cells to support osteoclastogenesis and to increase bone loss [107,149,150] (Figure 4). We and others have found that pharmacological modulation and genetic inactivation of Cnr2 influence the ability of tumour cells to cause osteolysis and to influence osteoblast – osteoclast crosstalk (Figure 5). Lozano-Ondoua *et al*. have found that the Cnr2 selective, non-psychoactive JWH015 reduced cancer-induce bone loss and cortical fracture in a murine bone cancer model of breast cancer [130,151]. Histological examination of longs bone from these mice revealed that this compound reduced skeletal tumour growth and reduced the level of the bone markers collagen type 1 cross-linked C-telopeptide (CTX), TRAcP5b and osteocalcin, indicative of significant inhibition of bone turnover. Further work by the same group albeit in a different mouse model of bone cancer has confirmed these findings and showed that AM1241 reduced sarcoma-induced osteolytic bone damage and fracture [151]. Whilst it is evident that these agents reduced cancer induced bone damage in the mouse models described, it is unclear whether these protective effects were due to reduction of skeletal tumour growth and/or osteoclast inhibition or stimulation of osteoblast differentiation as previously reported in animals models of osteoporosis [24,73]. It is also unclear if the agents tested in these studies act as partial/full inverse agonists or antagonists at Cnr2 and/or any other related receptor.

In recent studies carried out in our laboratories, we have examined the role of the skeletal Cnr2 receptor in the regulation of breast cancer-induced bone cell activity and osteolysis [76]. In our studies, we showed that mouse and human breast cancer cells and their derived factors enhanced RANKL-induced osteoclast formation and bone resorption *in vitro* and these effects were significantly enhanced in the presence of the Cnr2 selective agonists HU308 and JWH133 at concentration below 1μM and were inhibited by the Cnr2 selective inverse agonists AM630 or in cultures of pre-osteoclasts generated from Cnr2 deficient mice [76]. Then, we went on to demonstrate that pharmacological activation of Cnr2 in osteoblasts *in vitro* significantly enhanced the ability of osteotropic cancer cells to stimulate osteoclast formation. *In vivo* and *ex vivo* studies confirmed these findings and showed that activation of Cnr2 exacerbated breast cancer-induced osteolysis and bone loss by stimulating osteoclast

formation whereas genetic inactivation and pharmacological blockage of Cnr2 was inhibitory [76]. Mechanistic studies in cancer and bone cells revealed that Cnr2 activation regulates breast cancer cell – osteoblast – osteoclast differentiation and crosstalk by (a) enhancing breast cancer-induced RANKL/OPG ratio in osteoblasts and (b) breast cancer-related PI3-Akt activation in pre-osteoclasts. It is important to note that these effects were obtained at concentrations that failed to affect the viability and proliferation of osteoblasts, pre-osteoclasts and breast cancer cells, thereby excluding toxic effects. Evidence to date suggests that Cnr2 activation enhances bone cell activity and exacerbates breast cancer-induced osteolysis by a direct effect on bone cells, namely osteoblasts and osteoclasts (Figure 5). Thus, inhibition of Cnr2 receptor signaling under these conditions may have a potential therapeutic effect against cancer induced bone loss. It is important to note that these findings apply only to the bone cell-autonomous contribution of Cnr2 and do not exclude the strong inhibitory effects of Cnr2 ligands on tumour cell growth and proliferation *in vitro* and *in vivo* previously described by us [76] and other investigators [130,151].

3.1.3 Regulation of cancer-induced bone pain by Cnr2

Primary bone tumours and bone metastases are characterized by a debilitating pain (reviewed in [152,153]). The bone-mineralized matrix is densely innervated by sensory neurons and both Cnr1 and Cnr2 have been found to be expressed in peripheral neurons and in the immune cells such as microglia in the spinal cord (reviewed in [15]). Bone-infiltrating tumour cells secrete pro-inflammatory cytokines and factors, namely nerve growth factor (NGF) and endothelin 1 that are known to enhance pain perception (reviewed in [154]). Furthermore, a number of studies have shown that tumour cell growth and expansion in bone induces nerve compression and injury that in turn enhances cancer-induced bone pain [155-157]. Furthermore, pain, pro-angiogenic and pro-inflammatory mediators including IL-1β, TNFα and IL-6 produced by tumour-associated macrophages and monocytes play an important role in the pathogenesis of cancer-induced bone pain [154,158-160]. These findings together suggest that agents that suppress tumour growth, reduce inflammation,

inhibit osteoclastic bone damage and protect against nerve injury may be of value for the treatment pain associated with CIBD.

The endocannabinoid system is implicated in inflammatory, neuropathic and cancer-induced pain (reviewed in [23,151,161]). Secretion of endocannabinoids and expression of Cnr1 and Cnr2 receptors are upregulated in models of chronic pain [162]. A number of studies have shown that administration of phyto or synthetic cannabinoid receptor agonists exerted strong anti-nociceptive and anti-hyperalgesic effects in animal models of neuropathic [163] and inflammatory pain [164]. The clinical cannabis-based agent Sativex – which is known to activate both Cnr1 and Cnr2 - attenuated cancer-induced bone pain [161,165]. While these findings were encouraging, the adverse psychoactive effects caused by activation of cannabinoid receptors, in particular Cnr1, in the brain may limit the development of these agents for cancer treatment. This and the fact that long-term use of analgesics such as opiates is often associated with excessive bone loss [166] encouraged the development and testing of selective Cnr2 for the reduction of cancer-induced pain [23,167-171].

In 2010, Lozano and colleagues have reported that the Cnr2 selective ligand AM1241 reduced spontaneous and movement-evoked pain in a mouse model of sarcoma [151]. Later work by the same group showed that the Cnr2 selective JWH015 reduced spontaneous flinching and guarding in mice following intra-femoral injection of breast cancer cells [130], indicating a strong local analgesic effect in the model described. Recent work by Khasabova *et al*. confirmed the involvement of Cnr2 in the regulation of pain perception in cancer and showed that increased levels of the endocannabinoid 2AG by pharmacological inhibition of MAGL reduced tumour-evoked bone pain via a mechanism dependent on Cnr2 activation [172]. On the other hand, mechanistic studies showed that activation of Cnr2 signalling in immune cells and microglia reduced the levels of various pro-inflammatory mediators implicated in the regulation of pain [151,169,173,174]**.** Treatment with Cnr2 receptor agonists has also been found to enhance the release of endogenous opioid peptides that may contribute to the anti-nociception associated with Cnr2 activation [175,176]. Together, these findings suggest that selective targeting of the peripheral Cnr2 may have translational

benefits in identifying non-psychoactive, analgesic agents for the treatment of cancer induced pain.

4. Challenges and future direction

The majority of deaths from cancer following the failure of conventional therapies are a result of metastases. Although advances in early detection, chemotherapy and radiotherapy have significantly reduced cancer-related death, disease recurrence and acquired resistance to conventional therapies still remain major clinical problems. Cancer associated bone disease and pain are serious clinical complications of metastatic cancer that are often inadequately managed in patients with advanced disease [91,177,178]. To date, treatment of CIBD has focused on anti-osteoclast agents, such as bisphosphonates and Denosumab [129], which are known to reduce skeletal complications in patients with osteolytic metastatic disease but they are incompletely effective and lack a direct, significant effect on bone pain [179-182]. Thus, there is a need to identify additional therapeutic strategies that either alone or in combination with conventional anti-cancer agents suppress bone metastasis, reduce bone damage, halt recurrence, and attenuate pain in cancer patients with CIBD.

Endocannabinoids and their receptors play an important role in the regulation of tumorigenesis, metastasis and pain [6-10]. There has been increasing interest in targeting of the cannabinoid system in the treatment of cancer. Cannabinoid receptor agonists exert anti-emetic properties and have been used for the treatment of nausea and vomiting associated with cancer chemotherapy [57,183]**.** Cannabinoid receptor ligands also display analgesic effects, muscle relaxant properties and mood–elevating properties with a number of these ligands widely studied in patients with neuropathic and metastatic pain [57,183]. Cnr1 and Cnr2 are expressed in various tumours, and cannabinoid ligands reported to exert both stimulatory and inhibitory effects on cancer cell proliferation *in vitro* and tumour progression *in vivo* (Reviewed in [57,183]). In the last decade, the peripheral Cnr2 receptor has emerged as an attractive target for developing anti-cancer drugs. The most important rationale for the focus on Cnr2, is that ligands that selectively bind to Cnr2 would be expected to elicit less adverse psychoactive effects and tolerance associated with cannabinoid-based therapy

[23,176,184]. Therefore future studies are needed to compare the effects of cannabis clinical extracts such as Sativex and selective Cnr2 ligands on cancer metastasis. Systemic and prolonged treatment with selective Cnr2 agents may still cause adverse effects due to activation of Cnr2 and other opioid receptors in the peripheral [176] and albeit highly unlikely in the central nervous system. Thus, the development and testing of tumour-selective Cnr2 ligands that do not cross the blood-brain barrier would be of great benefit in eliminating the possibility of these adverse effects occurring. Such studies are timely and if successful, will (A) pave the way for future research that will advance our knowledge about the basic mechanism(s) by which the endocannabinoid system regulate cancer metastasis, (B) stimulate the research and development of a safer cannabis-based therapy for the treatment of metastatic cancer, (C) address a huge unmet clinical need, as metastasis is a major problem affecting advanced cancer patients and (D) provide policy makers with powerful tools to assess the science and therapeutic potential of cannabinoid-based therapy.

The Cnr2 receptor is implicated in the regulation of tumour – bone cell interactions and there are four main strategies that could potentially be pursued for targeting Cnr2 for the treatment of cancer associated bone disease; 1) reduction of metastasis to bone, 2) inhibition of tumour growth in bone, 3) suppression of cancer-induced osteoclast formation and osteolysis, and 4) stimulation of bone formation to aid with bone regeneration. Studies to date have indicated that agents that target Cnr2 in the skeleton have potential efficacy in the reduction of cancer-induced osteolysis and pain. However, the role of Cnr2 in the development and progression of bone metastases is yet not fully understood and very little research has so far been conducted on the direct effects of Cnr2 selective ligands on skeletal tumour growth, even though Cnr2 ligands have been studied as possible anti-tumour agents in primary cancers [183]. Furthermore, Cnr2 receptor plays an important role in bone formation but recent studies have failed to address the effects of Cnr2 selective ligands on osteoblast function in metastatic cancer. There are also other numerous unanswered questions and key issues that may limit the usefulness of Cnr2 selective agents as bone sparing drugs in cancer patients. For example, studies to date, including those conducted by our group [76], have failed to

address whether exposure to high concentrations and doses of Cnr2 selective ligands exert a compensatory expression and activity of other cannabinoids and/or their related receptors such as opioid and dopamine, which are known to dimerise with cannabinoid receptors [185,186]. In addition to Cnr2, other cannabinoid receptors, in particular Cnr1 and related receptors including GPR55, opioid and vanilloid receptors are implicated in the regulation of normal and cancer associated bone remodelling. Therefore more comprehensive studies in genetically modified animals that lack cannabinoid receptors and enzymes are needed to fully address their roles. Future studies should also take into account that pharmacological activation or antagonism of Cnr2 may lead to a complex reorganization of the fine-tuned endocannabinoid system that in turn result in a shift in the level of endocannabinoid balance, causing a transactivation of other related receptors such as Cnr1, GPR55 and vanilloid receptors which are known to influence bone cell activity and function [28,187]. The discovery of key intracellular pathways, particularly pivotal cytokine and immune cell receptors implicated in the regulation of bone – immune – cancer interactions may facilitate the advent of a new phase of Cnr2-based therapy for the treatment of cancer induced bone disease. Inhibition of elements involved in Cnr2 signalling has the potential to enhance the efficacy of radiotherapy and chemotherapy. The increase in our knowledge of these challenging and "thorny" issues may aid with the design of a better and more effective therapeutic strategy to selectively target Cnr2 signalling to suppress bone damage, alleviate bone pain and reduce skeletal tumour burden.

5. Conclusion

Targeting Cnr2 receptor has been a hot topic in cancer pain treatment in the last decades. A number of preclinical studies have demonstrated that the skeletal Cnr2 receptor plays a role in the regulation of tumour – bone cell interactions and preclinical studies showed that pharmacological targeting of Cnr2 are effective in reducing skeletal tumour burden, inhibiting osteolysis and attenuating bone pain in animal models of osteolytic bone disease. Notwithstanding the inherent limitations of the preclinical models used in these studies and the colossal challenges associated with the development and testing of peripherally-acting,

Cnr2-selective compounds, the present data indicate that small molecule ligands to Cnr2 could be of value for the treatment of skeletal complications associated with cancer. Future research and development of Cnr2-selective ligands will stimulate the development of safer cannabis-based therapy for the treatment of cancer. Thus, offering the prospect of identifying these agents, as novel, alternative to cannabis herbal extracts for the treatment of advanced cancer patients.

Acknowledgements

Aymen I. Idris would like to declare a financial interest on the development of cannabinoid ligands for the treatment of bone diseases.

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Figure legends

Figure. 1. Type 2 cannabinoid receptor expression. Type 2 cannabinoid receptor (Cnr2) is detected in a variety of tissues but it is predominantly expressed in cells of the immune system, localized area of the central nervous system and in bone cells.

Figure 2. The signal transduction pathways associated with type 2 cannabinoid receptor activation in cancer and bone cells. The majority of cancer-related effects associated with Cnr2 receptor are mediated by Gi/o-dependent activation of adenylyl cyclase activity (AC) and cAMP in cancer and host cells in tumour microenvironment. Reduced levels of intracellular cAMP levels prevent activation of transcription factors CREB, nuclear factor kappa B (NFκB) and AP1 by a PKA-dependent mechanism culminating in inhibition of expression of related genes. PKA also modulates the FAK/SRC/RHOA pathway and COX that regulate migration/invasion and angiogenesis. Ligand-induced activation of Cnr2 and Gi/o regulates the activity and expression of various proteins from the MAPK family including p38, JNK, ERK, PI3K/Akt and stimulates ceramide synthesis de novo in cancer cells, leading to activation of transcription factors involved in cell proliferation, cell-cycle progression and apoptosis- or autophagy-mediated cell death. Activation of Cnr2-mediated Akt in bone cells contributes to breast cancer induced osteoclast formation and osteolysis. Activation of Cnr2 receptor in cancer cells has been recently associated with the modulation of calcium and potassium channels and phospholipase C (PLC)-mediated release of calcium from the endoplasmic reticulum, leading to activation of NFATc1 transcription factor. Abbreviations; AC, adenylyl cyclase: cAMP, cyclic adenosine monophosphate; Gi, inhibitory class of G protein; PKA, protein kinase A; cAMP-dependent protein chinase; JNK, jun N- terminal kinase; p38, p38 mitogen-activated protein kinase; ERK, extracellular receptor kinase; PI3K, phosphoinositide 3-kinase; Akt, protein kinase B; mTORC, mammalian target of rapamycin complex 1; FAK, focal adhesion kinase; Src, sarcoma tyrosine kinase; RhoA, Ras homolog gene family, member A; COX-2, cycloxigenase-2;; CXCR4, chemokine receptor 4; CXCL12,

chemokine ligand 12; VEGF, vascular endothelial growth factor; MMP matrix metalloproteinase; PGE2, prostaglandin E2.

Figure 3. **The bone remodelling cycle during adulthood (a) and ageing (b).** Following mechanical stress or micro-damage, osteoclast precursors and mature osteoclasts initiate bone resorption by migrating to the site to be remodeled. The next major stage of the remodelling cycle involves the recruitment of osteoblast precursors to the site of resorption and deposition of new bone. Remaining osteoblasts are either buried within the newly deposited matrix as osteocytes or converted to lining cells that cover the majority of quiescent bone surface. Significant accumulation of adipocytes in the bone marrow cavity is often observed in aged skeleton (b). The newly remodeled bone enters into resting phase, where the quiescent lining cells await to be activated. oB, osteoblasts; oC, osteoclasts: adipo, adipocytes.

Figure 4. The bone remodelling cycle during osteolytic and osteoblastic cancer induced bone disease. (a) Malignant tumour cells that colonize bone cause bone osteolytic lesions by secreting factors that act directly, on osteoclasts and their precursors, or indirectly, on osteoblasts, thereby enhancing osteoclast formation and activity. As a consequence of increased resorption by osteoclasts, released bone-derived factors further enhance tumour cell proliferation, thereby exacerbating osteolytic bone loss. (b) Osteoblastic bone lesions are caused by excessive stimulation of osteoblast proliferation, differentiation and function by cancer cells that often leads to the formation of abnormal and disorganized new bone. This phase is often followed by an increase in osteoclast number and bone resorption. Parathyroid hormone-related peptide (PTHrP), tumor necrosis factor –alpha (TNF-α), TGF-β, prostaglandin E2 (PGE2), bone morphogenetic protein (BMPs), platelet derived growth factors (PDGF), endothelin-1 (ET-1), insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF) and interleukins (ILs), matrix metalloproteinases (MMPs).

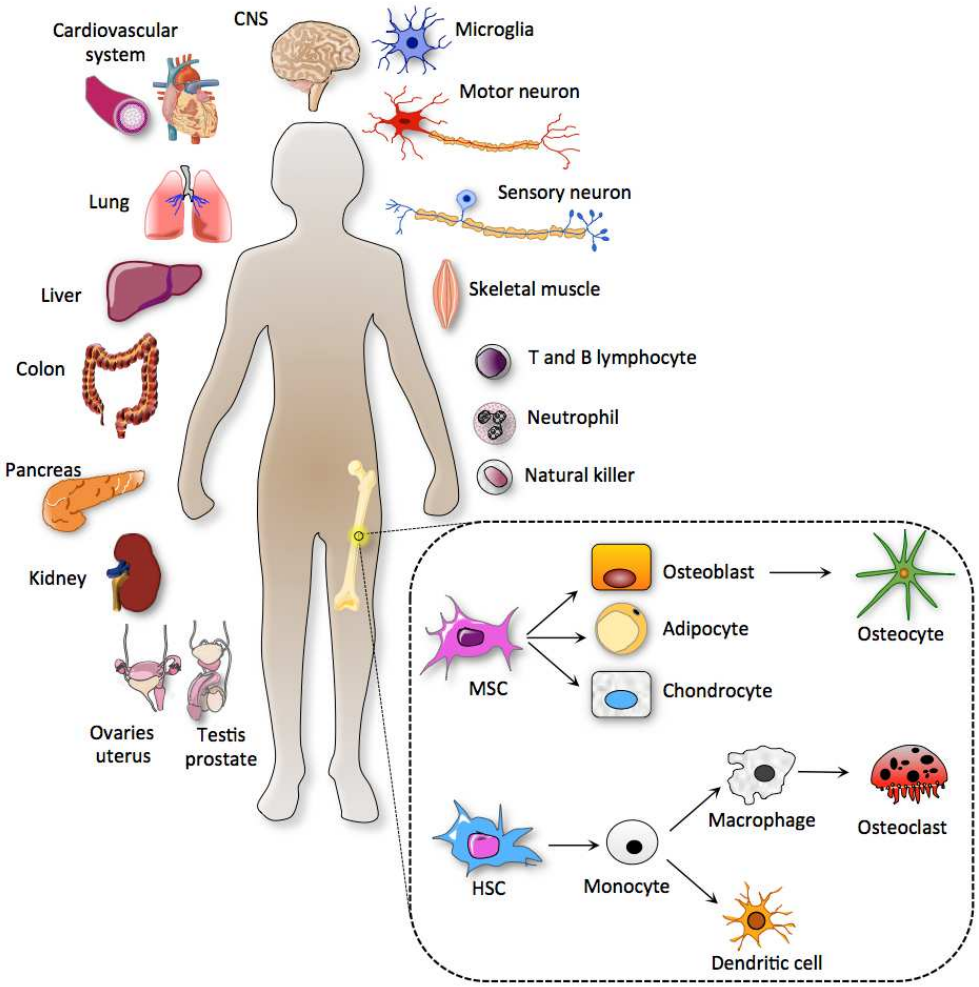
Figure 5. Current model of regulation of cancer – bone cell interactions by Cnr2. Pharmacological activation of Cnr2 *in vivo* inhibits osteoclast number, increase osteoblast number, reduces osteolysis and attenuates bone pain in mouse preclinical models of cancer.

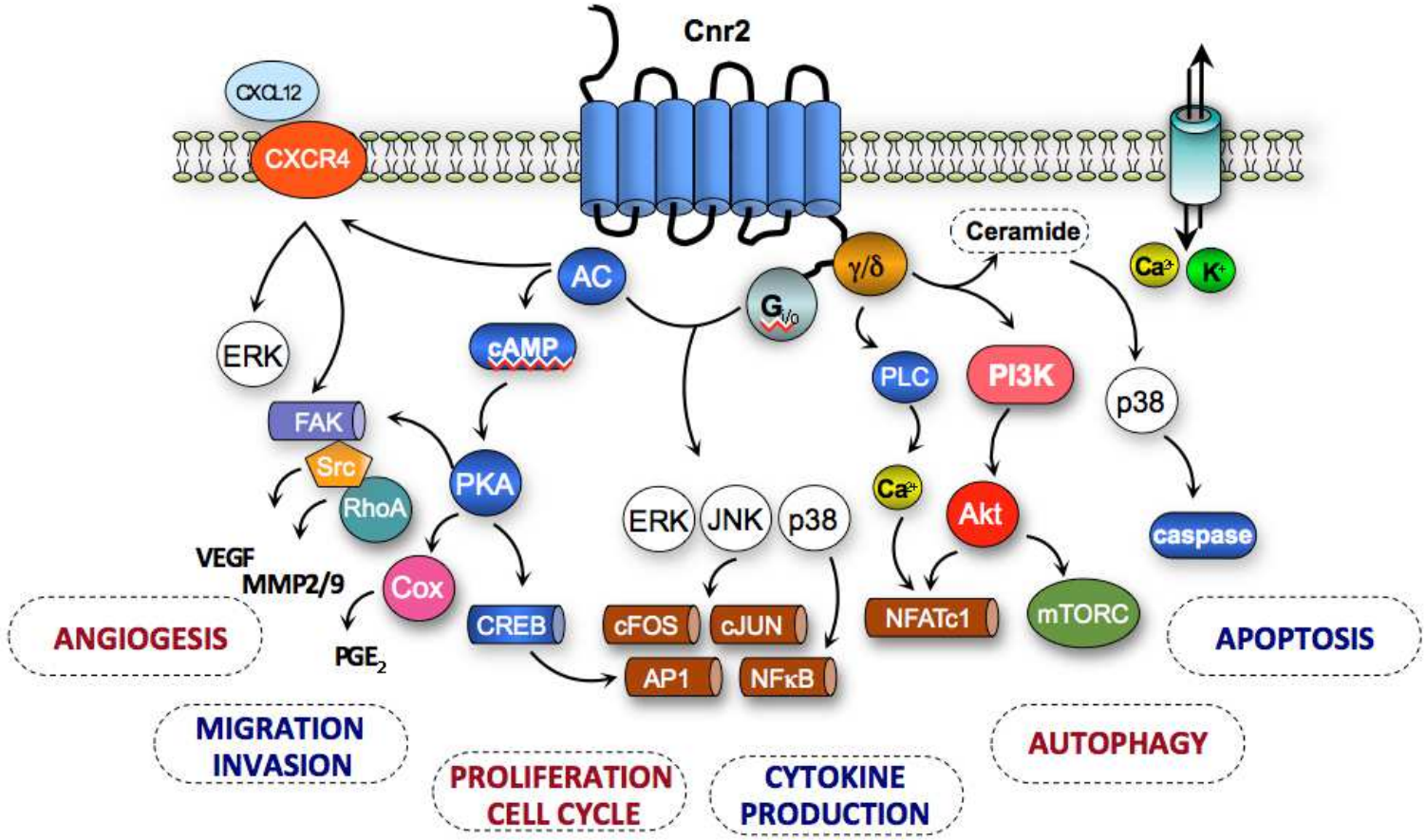
In vitro, a biphasic effect of Cnr2 activation has been observed. Low concentrations of Cnr2 agonists increase osteoclast formation and activity without affecting tumour cell proliferation, whereas high concentrations exert anti-resorptive and anti-tumour effects. Abbreviations; oB, osteoblasts; oC, osteoclasts, Tm, cancer cells, nM, nano-Molar, μM, microMolar.

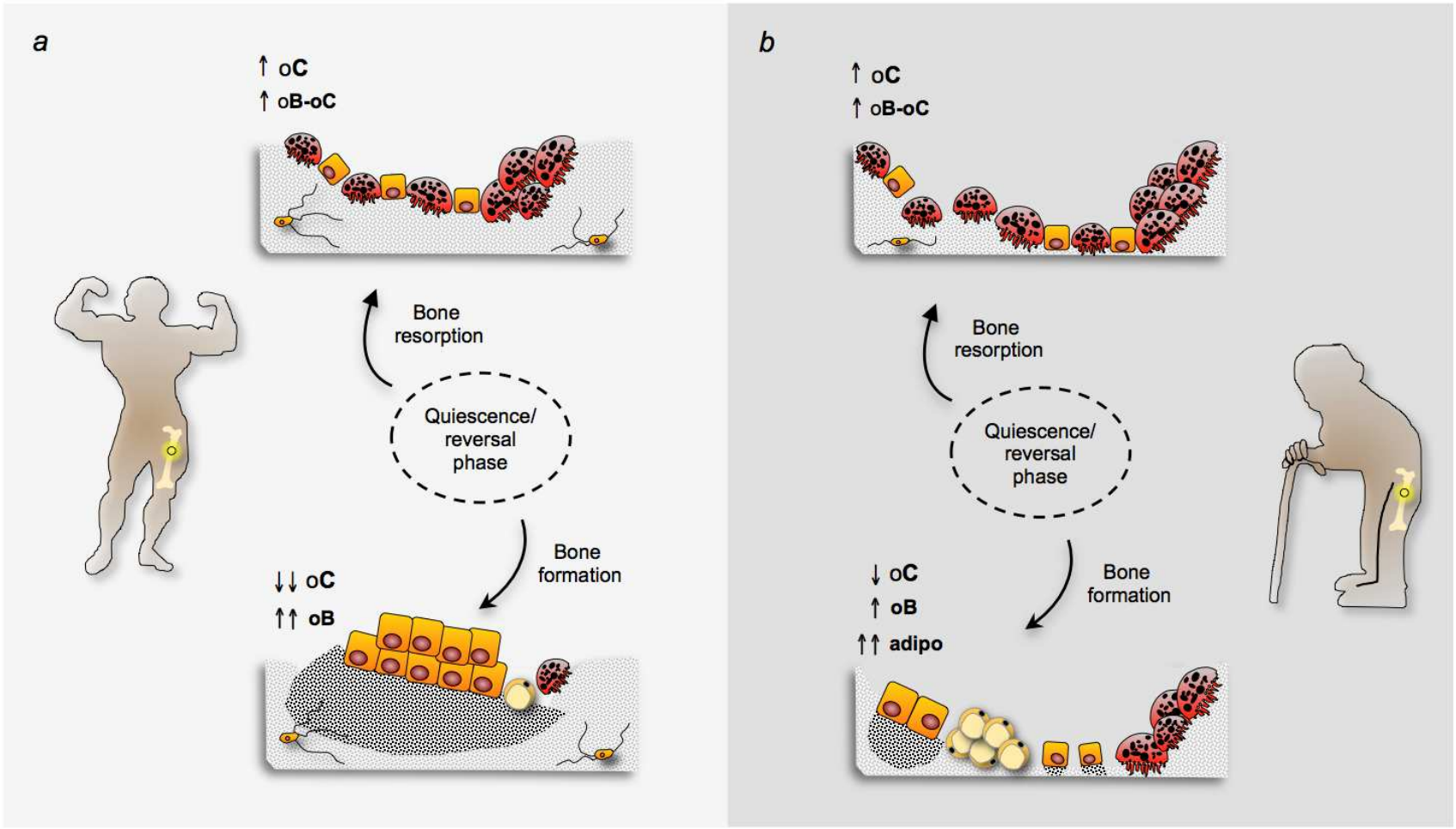
Table 1. The role of cannabinoid receptor ligands in the Cnr2-mediated regulation of osteoclast, osteoblast and cancer *in vitro* and *in vivo*.

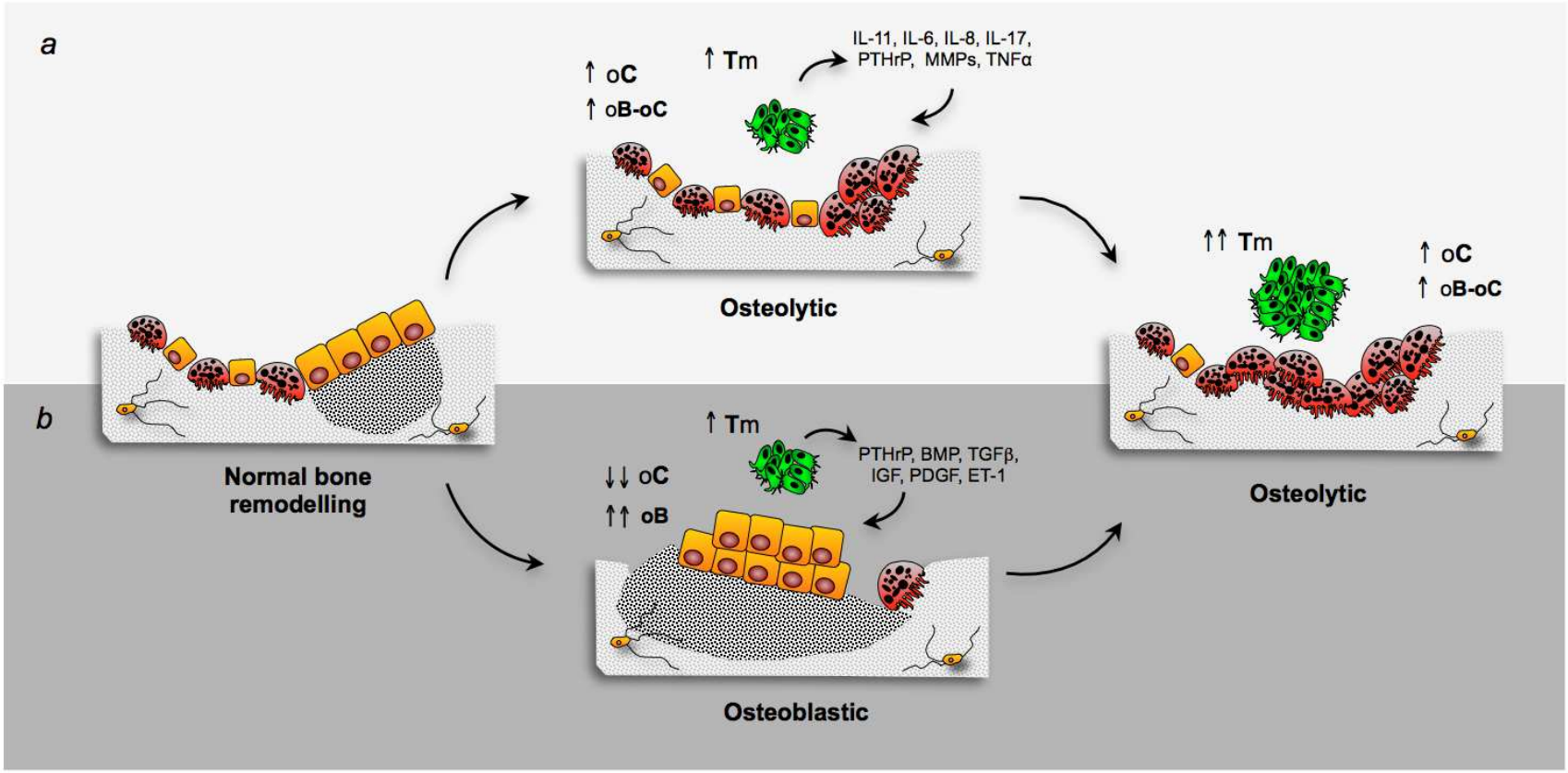
			Bone metabolism			Cancer		
	Ligand	**Receptor**	**Oc. Number**	**Oc. Activity**	**Ob. Number**	**Tm. Growth**	**Osteolysis**	**Pain**
Endocannabinoid	AEA	Cnr2/Cnr1/GPR55	↑	↑	↑	↓↓	-	↓
	2-AG	Cnr2/Cnr1/GPR55	↑	↑	↑	↓	↓	-
Phytocannabinoids	∆9-THC	Cnr2/Cnr1	-	-	-	↓↓↑↑	-	↓
	Cannabidiol	Cnr2/Cnr1/ TRPV1	↑	↓↓	-	↓↓	-	↓
Agonists	HU308	Cnr2	↑↓↓	↓	↑↑	↑↓	↑*	-
	JWH133	Cnr2	↑	↑	↑	↑↓↓	↓↓↑*	-
	JWH139	Cnr2	-	-	-	↓	-	-
	JWH015	Cnr2	-	↑	↑	↓↓	↓	↓
	AM1241	Cnr2	-	-	-	↓	↓	↓
	WIN55,212-2	Cnr2/Cnr1	-	-	↑	↓↓	-	↓
	CP55,940	Cnr2/Cnr1	↑↓	↑	↑	↓	-	↓
Antagonists/ Inverse agonist	AM630	Cnr2	↓↓↑	↓	-	-	↓*	-
	SR144528	Cnr2	↓	↓	-	-	-	-

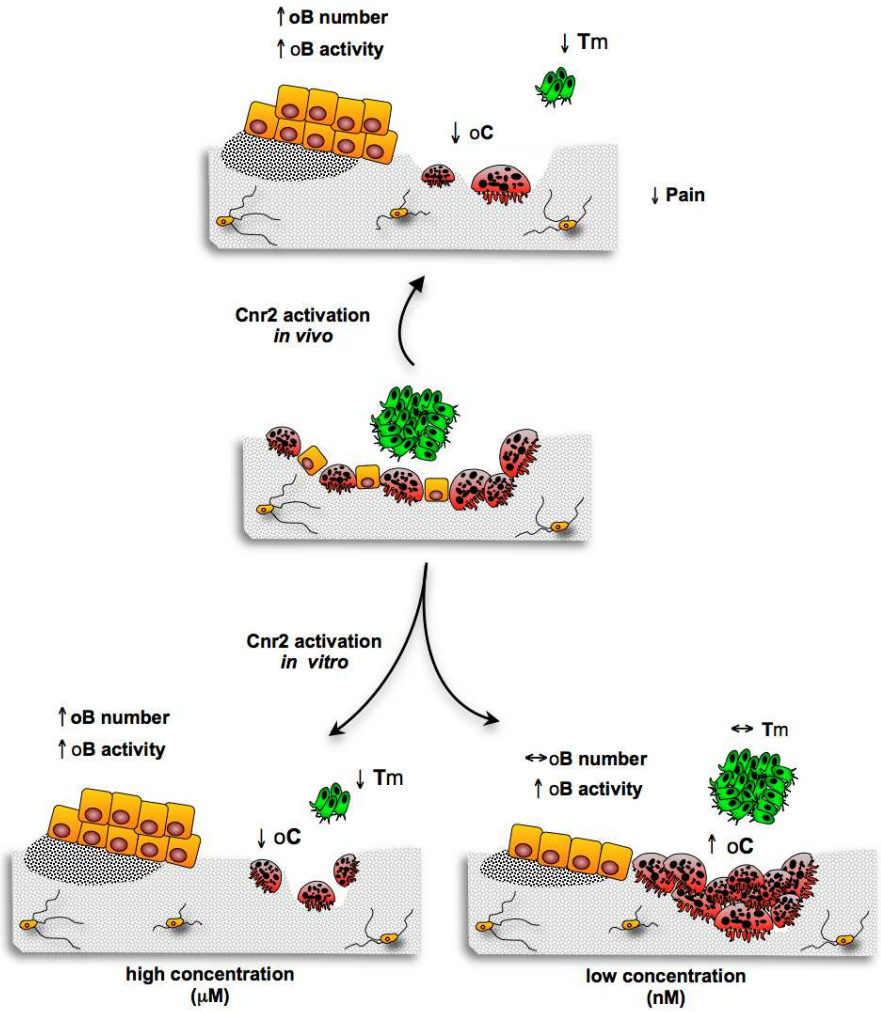
The data presented in this table are assembled from references [21,24,25,73,76,81,85,188]. ↑, increase; ↓, decrease; -, non tested:* *ex-vivo* models. Black and red arrows denote *in vitro* and *in vivo* data, respectively. Abbreviations; Oc., osteoclast, Ob., osteoblast, Tm. Tumour cell.

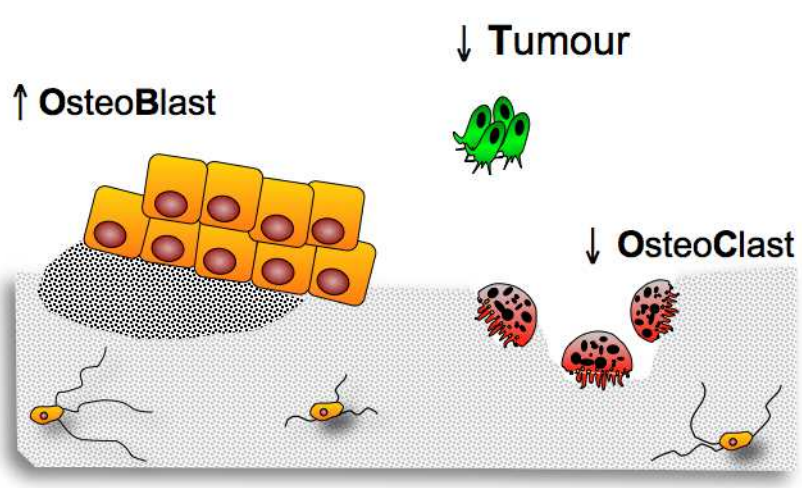

Cardiovascular system
CNS
Microglia
Motor neuron
Lung
Sensory neuron
Liver
Skeletal muscle
Colon
T and B lymphocyte
Neutrophil
Pancreas
Natural killer
Kidney
Osteoblast
Adipocyte
Osteocyte
MSC
Chondrocyte
Ovaries uterus
Testis prostate
Macrophage
Osteoclast
HSC
Monocyte
Dendritic cell


Cnr2
CXCL12
CXCR4
AC
γ/δ
Ceramide
Ca2+
K+
ERK
cAMP
PLC
PI3K
p38
FAK
Src
RhoA
PKA
Ca2+
Akt
caspase
ERK
JNK
p38
VEGF
MMP2/9
Cox
CREB
cFOS
cJUN
NFATc1
mTORC
ANGIOGESIS
PGE2
AP1
NFκB
APOPTOSIS
MIGRATION
INVASION
AUTOPHAGY
PROLIFERATION
CELL CYCLE
CYTOKINE
PRODUCTION

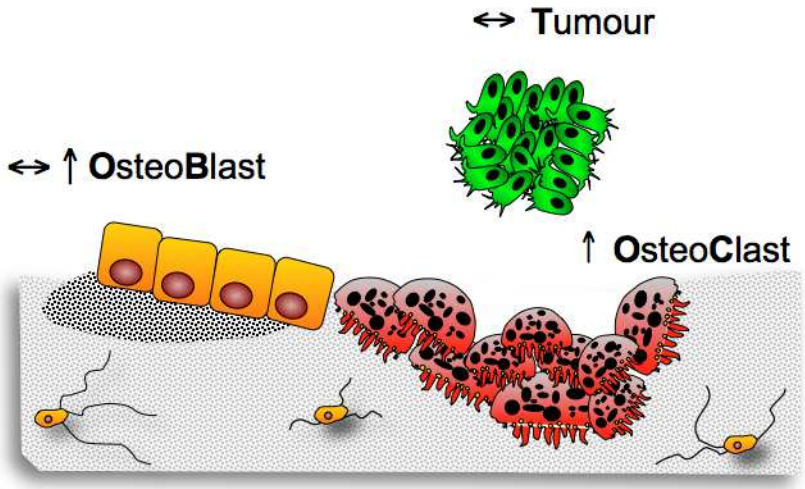

a
↑ oC
↑ oB-oC
Bone resorption
Quiescence/ reversal phase
Bone formation
↓↓ oC
↑↑ oB
b
↑ oC
↑ oB-oC
Bone resorption
Quiescence/ reversal phase
Bone formation
↓ oC
↑ oB
↑↑ adipo


a
↑ oC
↑ oB-oC
↑ Tm
IL-11, IL-6, IL-8, IL-17,
PTHrP, MMPs, TNFα
Osteolytic
↑↑ Tm
↑ oC
↑ oB-oC
Osteolytic
b
Normal bone
remodelling
↑ Tm
PTHrP, BMP, TGFβ,
IGF, PDGF, ET-1
↓↓ oC
↑↑ oB
Osteoblastic


↑oB number
↑oB activity
↓ Tm
↓ oC
↓ Pain
Cnr2 activation
in vivo
Cnr2 activation
in vitro
↑oB number
↑oB activity
↓ Tm
↓ oC
high concentration
(μM)
↔oB number
↑ oB activity
↔ Tm
↑ oC
low concentration
(nM)



Cnr2 activation
High concentration
Osteoprotection



Cnr2 activation
Low concentration
Osteolysis