

REVIEW ARTICLE

Cannabinoids and the skeleton: From marijuana to reversal of bone lossITAI BAB¹, ANDREAS ZIMMER² & EITAN MELAMED¹¹Bone Laboratory, the Hebrew University of Jerusalem, Jerusalem, Israel, and ²Institute of Molecular Psychiatry, University of Bonn, Bonn, Germany**Abstract**

The active component of marijuana, Δ^9 -tetrahydrocannabinol, activates the CB1 and CB2 cannabinoid receptors, thus mimicking the action of endogenous cannabinoids. CB1 is predominantly neuronal and mediates the cannabinoid psychotropic effects. CB2 is predominantly expressed in peripheral tissues, mainly in pathological conditions. So far the main endocannabinoids, anandamide and 2-arachidonoylglycerol, have been found in bone at 'brain' levels. The CB1 receptor is present mainly in skeletal sympathetic nerve terminals, thus regulating the adrenergic tonic restraint of bone formation. CB2 is expressed in osteoblasts and osteoclasts, stimulates bone formation, and inhibits bone resorption. Because low bone mass is the only spontaneous phenotype so far reported in CB2 mutant mice, it appears that the main physiologic involvement of CB2 is associated with maintaining bone remodeling at balance, thus protecting the skeleton against age-related bone loss. Indeed, in humans, polymorphisms in *CNR2*, the gene encoding CB2, are strongly associated with postmenopausal osteoporosis. Preclinical studies have shown that a synthetic CB2-specific agonist rescues ovariectomy-induced bone loss. Taken together, the reports on cannabinoid receptors in mice and humans pave the way for the development of 1) diagnostic measures to identify osteoporosis-susceptible polymorphisms in *CNR2*, and 2) cannabinoid drugs to combat osteoporosis.

Key words: Bone, cannabinoid, endocannabinoid, marijuana, osteoporosis**Introduction**

The marijuana plant *Cannabis Sativa* has been cultivated for thousands of years for medical and recreational use in the form of marijuana or hashish. Its psychoactive effects have made it the most common drug of abuse. However, it affects not only the brain but virtually every organ system in the body. We now know that the active component of marijuana, Δ^9 -tetrahydrocannabinol (THC), acts on two distinct receptors that are distributed throughout the body, only one of which mediates the psychotropic effects. These receptors respond to endogenous ligands, termed endocannabinoids, with THC just mimicking the activity of these physiological activators. The endocannabinoids are produced and degraded by specific enzymes. Together, the receptors, ligands, and enzymes comprise the endocannabinoid system. The on-going discovery of this system during the last two decades and its relevance for many organ systems has fueled extensive research and tremendous interest from

pharmaceutical companies for potential therapeutic applications. Progress in this field has exploded in the last decade. There is a huge literature, growing by the day, investigating the endocannabinoid system in neural and non-neural tissues. Numerous excellent reviews have appeared in the last several years that treat the history, biochemistry, pharmacology, and therapeutic potential of this system (1–8).

In ancient times, *Cannabis* was used therapeutically to relieve pain, reduce inflammation, and as a sedative. It was also used extensively to treat migraine headaches and ulcers. It is now well established that THC produces numerous beneficial effects, including analgesia, appetite stimulation, nausea reduction, and reduction of intraocular pressure. THC also affects fertility, short-term memory, tumor growth, and motor co-ordination (9,10). The therapeutic use of THC has been hampered by psychotropic effects that have prevented general acceptance by the Federal Drug Administration (FDA). MarinolTM, a synthetic

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Key messages

- Several key components of the endocannabinoid system have been identified in bone.
- The main physiologic involvement of CB2 (type 2 cannabinoid receptor) is associated with maintaining bone remodeling at balance.
- CB2 agonists are possible candidates for a combined antiresorptive and anabolic therapy for osteoporosis.

THC in sesame oil, is the only FDA-approved cannabinoid agonist for use in the US. It is prescribed as an appetite stimulant in AIDS, gastric by-pass, and chemotherapy patients and also as an antiemetic for chemotherapy. Sativex™ was approved by Health Canada in 2005 to relieve pain and spasticity in multiple sclerosis. It is a sublingual spray made by blending two of the main active ingredients of cannabis, THC and cannabidiol (CBD), and used for the relief of neuropathic pain. It has been recently (December 2008) approved by the FDA for stage III clinical trials in the US. An antagonist of the type I cannabinoid receptor (CB1), marketed as Acomplia™ by Sanofi Aventis, was approved for use as an antiobesity drug in the European Union in 2006. It also blocks the weight-gain associated with nicotine withdrawal, reduces visceral fat content, and lowers LDL levels. However, its marketing in Europe as well as stage III clinical trials of Acomplia™ in the US have been recently halted due to a 2-fold increase in psychiatric (depression, anxiety, irritability) and gastrointestinal (nausea) side-effects (11).

Recently, there has been a rapidly growing interest in the role of cannabinoids in the regulation of skeletal remodeling and bone mass, addressed in basic, translational, and clinical research. A recent citation search revealed as many as 80 publications addressing the skeletal cannabinoid system since the first publications in 2005 (12,13). This review focuses on the skeletal cannabinoid system as a therapeutic target for patients with osteoporosis and other skeletal deficits.

Skeletal remodeling

Bone structure displays sequential stages throughout life, comprising 1) a rapid skeletal growth phase accompanied by accrual of peak bone mass; 2) a steady state phase whereby bone mass remains constant; and 3) age-related bone loss (14). These changes are the consequence of a continuous process

Abbreviations

2-AG	2-arachidonoylglycerol
β2AR	β2-adrenergic receptor
BMP	bone morphogenetic protein
DAGL	diacylglycerol lipase
FAAH	fatty acid amide hydrolase
IL-6	interleukin 6
MAPK	mitogen-activated protein kinase
M-CSF	macrophage colony-stimulating factor
NAPE-PLD	N-acyl phosphatidyl ethanolamine phospholipase D
OGP	osteogenic growth peptide
OPG	osteoprotegerin
OVX	ovariectomy
PTH	parathyroid hormone
RANKL	receptor activator of NFκB ligand
SNP	single nucleotide polymorphism
THC	Δ ⁹ -tetrahydrocannabinol
TRPV1	transient receptor potential vanilloid type 1 receptor

of resorption and formation of the mineralized matrix, referred to as bone remodeling. The remodeling process occurs concomitantly in multiple foci that encompass approximately 5% of trabecular, endosteal, and osteonal surfaces (15). The remodeling cycle in individual foci consists of a relatively rapid (i.e. a few weeks in humans) resorption of pre-existing mineralized matrix by a specific hematopoietic, monocyte-derived cell type, the osteoclast (16). It is then followed by a slower (i.e. a few months) stage of bone formation by another bone-specific, fibroblast-like cell type, the osteoblast (17). Different foci present different phases of the remodeling cycle. Healthy adults retain an overall balance between bone resorption and formation. The significance of balanced bone remodeling is demonstrated by osteoporosis, the most common metabolic bone disease in developed societies, which results from a net increase in bone resorption and bone loss, weakening of the skeleton, and increased fracture risk.

The co-ordinated occurrence of multiple remodeling sites is suggestive of a complex hierarchical regulation consisting of local, autocrine/paracrine, and systemic endocrine regulatory systems (18). Indeed, numerous studies have demonstrated paracrine control of osteoclast formation and activity by factors such as receptor activator of NFκB ligand (RANKL), osteoprotegerin (OPG), macrophage colony-stimulating factor (M-CSF) and interleukin 6 (IL-6), which are derived from neighboring stromal cells, including osteoblasts and their precursors (19–24). Locally, osteoblasts are regulated mainly by bone morphogenetic proteins

(BMPs) and Wnts (25,26). Systemically, it is well established that depletion of gonadal hormones in females and males favors bone loss (27–29). In addition, parathyroid hormone (PTH) (30,31), calcitonin (32), insulin-like growth factor I (IGF-I) (33), osteogenic growth peptide (OGP) (34), and duodenum-derived serotonin (35) are involved in the control of bone formation. Bone remodeling is also subject to a central control by hypothalamic leptin and neuropeptide Y signaling as well as downstream sympathetic signaling through osteoblastic β 2-adrenergic receptors (β 2AR) (36–38). Furthermore, imbalances in bone remodeling, previously attributed to excessive thyroid activity and estrogen depletion, may result from the interaction between thyroid and follicular stimulating hormones and their receptors, which are expressed in bone cells (39,40).

Cannabinoid receptors and ligands

The actions of cannabinoids are mediated mainly through the activation of G protein-coupled cannabinoid CB1 and CB2 receptors (41). CB1 and CB2 share 44% overall identity (68% identity for the transmembrane domains). CB1 is perhaps the most abundantly expressed G protein-coupled receptor in the central nervous system. It is also present in peripheral neurons and the gonads and to some extent in several other peripheral tissues. CB2 is expressed in the skeleton, immune system, cirrhotic liver, arteriosclerotic plaques, inflamed gastrointestinal mucosa, and in glial and inflammatory cells in pathological brain conditions (42,43). That CB1 and CB2 are not functionally identical is demonstrated by the presence of cannabinoid agonists and antagonists with distinct binding specificities to either receptor (44,45). Both receptors signal via the $G_{i/o}$ subclass of G proteins, inhibiting stimulated adenylyl cyclase activity. Further downstream, the CBs induce the activation of p42/44 mitogen-activated protein kinase (MAPK), p38 MAPK, c-Jun N-terminal kinase, AP-1, the neural form of focal adhesion kinase, protein kinase B, and K^+ and Ca^{2+} transients (46). It has been proposed that GPR55 and transient receptor potential vanilloid type 1 receptor (TRPV1) are also involved in endocannabinoid triggering of these events (47–49).

The main CB1 and CB2 endogenous ligands are *N*-arachidonylethanolamine (AEA or anandamide) and 2-arachidonoylglycerol (2-AG) (50,51). Anandamide is present in a variety of tissues, such as the brain, kidney, liver, spleen, testis, uterus, and blood, in picomol/g concentrations, with the highest levels reported in the central nervous system. The low

anandamide concentrations have been attributed to low substrate (arachidonic acid esterified at the 1-position) levels for this pathway and/or the short anandamide half-life *in vivo* ($t_{1/2} < 5$ min) (52,53). Anandamide is biosynthesized through *N*-acyl phosphatidyl ethanolamine phospholipase D (NAPE-PLD)-dependent and -independent pathways (54). The main anandamide-degrading enzyme is fatty acid amide hydrolase (FAAH), a membrane-associated serine hydrolase enriched in the brain and liver (55). In general, the tissue distribution of 2-AG is similar to that of anandamide. However, its concentration in is 300–1,000 higher (ng/g range). 2-AG production has been demonstrated in the central nervous system, platelets, and macrophages, especially in response to stimulation by inflammatory agents such as lipopolysaccharide (56,57). It is generated from arachidonic acid-enriched membrane phospholipids, such as inositol phospholipids, through the combined actions of phospholipase C and diacylglycerol lipases (DAGL α and DAGL β) (58,59). It has been proposed that, like other monoacylglycerols, 2-AG is metabolized by a monoacylglycerol lipase (60).

A couple of striking observations lead us to assess the occurrence and role of a skeletal endocannabinoid system. One is that bone formation and bone mass, as well as the central production of at least one major endocannabinoid, 2-AG, are subject to negative control by leptin (61). The second observation is that traumatic brain injury enhances both bone formation (62,63) and central 2-AG production (64).

The skeletal endocannabinoid system

Several key components of the endocannabinoid system have been identified in bone. The main endocannabinoids, anandamide and 2-AG, are present in this tissue in pmol/g and nmol/g concentrations, respectively, levels similar to those found in the brain. Because the blood endocannabinoid levels are several orders of magnitude lower than those found in bone, it is very likely that anandamide and 2-AG are synthesized locally in the skeleton (65). Indeed, both ligands are produced by osteoblasts and osteoclasts in culture. In addition, diacylglycerol lipases (DAGLs) α and β , enzymes critically involved in 2-AG biosynthesis, are expressed in osteoblasts, osteocytes, and bone-lining cells (66). The respective anandamide biosynthetic and degrading enzymes, NAPE-PLD and FAAH (55), are also expressed in bone cells ((67) and our unpublished results). Although both 2-AG and anandamide are perceived as non-selective agonists of CB1 and CB2,

findings in bone and bone cell cultures suggest differential effects of these ligands. While 2-AG activates CB1 in the sympathetic nerve terminals following a single or chronic administration to mice, thus stimulating bone formation ((66) and our unpublished results), it has no effect on osteoblasts and may even act as an inverse agonist in these cells (66,68). Like the CB2-selective agonist, anandamide stimulates *in vitro* osteoblast proliferation (our unpublished results). In addition, the number of osteoclasts in culture is increased by a direct challenge with anandamide (13) or through the action of the FAAH inhibitor URB597 that leads to increased anandamide levels endogenously (67). It remains to be seen whether these actions of anandamide are mediated by CB1, CB2, GPR55, and/or TRPV1.

Effects on bone cell differentiation and activity

Activation of CB2 has different effects in early osteoblast progenitors and in more mature osteoblastic cells. In the early precursors, represented by bone-marrow-derived, partially differentiated osteoblastic cells that show limited CB2 expression, the specific CB2 agonist HU-308 (45), but not the specific CB1 agonist noladin ether (69), triggers a G_i protein-mediated mitogenic effect and consequent expansion of the preosteoblastic pool. *Ex vivo* osteoblastic colony (CFU-Ob) formation by bone-marrow stromal *cb2^{-/-}* cells is markedly diminished, whereas CFU-Ob formation by wild-type cells is stimulated by HU-308 (70,71). In mature osteoblastic cells, represented by the MC3T3 E1 cell line, the same ligand stimulates osteoblast-differentiated functions such as alkaline phosphatase activity and matrix mineralization (12,70). Hence, CB2 signaling is involved in several regulatory, pro-osteogenic processes along the osteoblast lineage.

In mouse bone-marrow-derived osteoclastogenic cultures and in the RAW 264.7 cell line, CB2 activation inhibits osteoclast formation by restraining mitogenesis at the monocytic stage, prior to incubation with RANKL. It also suppresses osteoclast formation by repressing RANKL expression in osteoblasts and osteoblast progenitors (70). Likewise, it has been recently shown that the cannabinoid receptor agonist ajulemic acid also suppresses osteoclastogenesis (72). By contrast, another study reported the stimulation of osteoclast formation and bone resorption by cannabinoid receptor agonists and their inhibition by antagonists (13,73). These allegedly paradoxical effects may be species- and/or agonist-dependent, as in human osteoclasts and other cells anandamide has been shown to activate

also TRPV1 (49). TRPV1 activation in the human osteoclasts and osteoclast precursors enhances osteoclast formation and activity (67) and may modify the effect of selective CB2 agonists. In addition to CB2, low levels of CB1 mRNA have been also reported in bone cell cultures (13,67,70). However, before drawing any conclusions related to the functional significance of CB1 in bone cells, its expression in these cells must be further evaluated at the protein level and *in vivo* using conditional gene ablation.

Skeletal phenotypes of cannabinoid receptor-deficient mice

Cannabinoid receptor mutant mice have been used to assess the physiologic role of CB1 and CB2 in the control of bone mass. In the case of CB1, the skeletal phenotype depends on the mouse strain and/or the construct used for gene mutation. In one CB1-deficient line, back-crossed to CD1 mice (CD1^{CB1^{-/-}}), the N-terminal 233 codons of the *CNR1* gene (which encodes CB1) were ablated (74). The effect of this mutation shows a clear gender disparity. Females have normal trabecular bone with a slight cortical expansion, whereas male CD1^{CB1^{-/-}} mice exhibit high bone mass (75). Sexually mature CD1^{CB1^{-/-}} mice of either gender display normal bone formation and resorption parameters, suggesting that the male phenotype is acquired early in life, during the developmental phase when peak bone mass is determined. A similar male phenotype was reported when mice carrying the same mutated *CNR1* gene were further back-crossed to Biozzi ABH mice (13,65,76). In the second line, back-crossed to C57BL6J mice (C57^{CB1^{-/-}}), almost the entire protein-encoding sequence was removed (77). Both male and female C57^{CB1^{-/-}} have a low bone mass phenotype accompanied by increased osteoclast counts and decreased bone formation rate (75). Our recent findings suggest that CB1 controls osteoblast function by negatively regulating norepinephrine (NE) release from sympathetic nerve terminals in the immediate vicinity of these cells. NE suppresses bone formation by binding to osteoblastic β2AR (38); this suppression is alleviated by activation of sympathetic CB1 (66).

Animals with a *CNR2*-mutated gene (which encodes CB2) have a gender-independent skeletal phenotype. During their first 2–3 months of life, *CNR2^{-/-}* mice accrue a normal peak trabecular bone mass but later display a markedly enhanced age-related bone loss; their trabecular bone volume density at 1 year of age is approximately half compared to wild-type controls (70). Reminiscent

of human postmenopausal osteoporosis (78), the *CNR2*^{-/-} mice have a high bone turn-over with increases in both bone resorption and formation which are at a net negative balance (70). Importantly, low bone mass is the only spontaneous phenotype so far reported in these mice. Hence, because healthy CB2 mutant mice are otherwise normal, it appears that the main physiologic involvement of CB2 is associated with maintaining bone remodeling at balance.

The endocannabinoid system as a target for antiosteoporotic therapy and osteoporosis risk assessment

Unlike CB1, CB2 is not associated with the cannabinoid psychotropic effects. Therefore, CB2-specific ligands could offer an opportunity to prevent and/or rescue bone loss while avoiding the psychological adverse effects of cannabinoids. Indeed, the specific, non-psychoactive CB2 agonist HU-308 attenuates bone loss induced by estrogen depletion in ovariectomized (OVX) animals using either 'preventive' or 'rescue' protocols (65,70). In the preventive approach, HU-308 administration commenced immediately after OVX. To assess reversal of bone loss, the drug was given beginning 6 weeks post-OVX to allow for bone loss to occur. In either protocol, the cannabinoid treatment consisted of daily intraperitoneal injections for 4–6 weeks. The attenuation of bone mass reflected both inhibition of bone resorption and stimulation of bone formation. Hence, CB2 agonists are possible candidates for a combined antiresorptive and anabolic therapy for osteoporosis.

Interestingly, marijuana smoke inhalation was recently reported to inhibit endosseous implant anchorage in rats, negatively affecting both the bone-implant contact and peri-implant bone (79). This is not necessarily in contradiction to the bone anabolic activity of well defined cannabinoid receptor agonists, as marijuana contains a mixture of biologically active phytocannabinoid whose skeletal effects have not been tested yet. In addition, the peri-implant healing process may differ considerably from remodeling of the non-traumatized skeleton and thus respond differently to cannabinoids. Another potentially confounding issue is the non-selectivity/non-specificity of many cannabinoid ligands, either endogenous, plant-derived, or synthetic. The skeletal relevance of this issue has been recently demonstrated in a study showing that the 'so-called' CB2-selective inverse agonist AM360 at a daily dose of 0.1 mg/kg prevented OVX-induced bone loss in wild-type but not in *CNR2*^{-/-} mice,

therefore indicating CB2 selectivity at this low dose. However, the same preparation was equally effective in preventing bone loss in wild-type and *CNR2*^{-/-} mice at higher doses (73). Hence, in the skeleton, and probably elsewhere, cannabinoid ligands may have CB1- and/or CB2-independent effects, depending on concentrations or doses used.

Polymorphisms in the human *CNR1* and *CNR2* loci were studied to assess the cannabinoid receptors as targets for the risk assessment and treatment of osteoporosis. The *CNR1* locus is located on chromosome 5q15 and encompasses a single coding exon that is preceded by several non-coding 5' exons, indicating a complex transcriptional regulation of this gene by different promoters (80,81). The *CNR2* locus is located on chromosome 1p36. This genomic region and its mouse ortholog on chromosome 4 have been linked to bone mineral density (BMD) and osteoporosis in several independent association analyses (82–84). However, these analyses did not consider *CNR2* as a potential candidate gene. Like *CNR1*, the *CNR2* gene also consists of a single coding exon, which is preceded by a non-coding upstream exon.

Thus far, two genetic association studies have been reported dealing with the relationship between polymorphisms in *CNR* genes and osteoporosis. The first study was carried out in a French Caucasian sample comparing postmenopausal osteoporotic women with a low bone mineral density (BMD) and age-matched healthy controls (85). Analysis of four single nucleotide polymorphisms (SNPs) spanning nearly 20 kb around the CB1 coding exon revealed no significant association with the osteoporosis phenotype, suggesting that the *CNR1* locus does not have a major role in this sample.

In the *CNR2* gene a total of 26 SNPs were analyzed, spanning approximately 300 kb around the *CNR2* locus. Several of these SNPs showed a significant association with the disease phenotype, suggesting that *CNR2* polymorphisms are important genetic risk factors for osteoporosis. The most significant *P*-values for allele and genotype associations were observed with SNPs located within the CB2 coding region (*P*=0.0014 and *P*=0.00073, respectively). Furthermore, when BMD at the lumbar spine was analyzed as a quantitative trait, highly significant differences were found in BMD between individuals carrying different SNPs in the CB2 coding region. Indeed, sequencing the CB2 coding exon in all patients and controls identified two missense variants, Gln63Arg and His316Tyr, with the Arg63 variant being more common in the osteoporotic patients than in the healthy controls (85). Taken together, these findings suggest that a

common variant of the CB2 receptor contributes to the etiology of osteoporosis in humans.

The second is a prospective study, which analyzed several candidate quantitative trait loci in BMD, including *CNR2*, in a cohort of 1,110 women and 1,128 Japanese men, 40–79 years of age (86). For the *CNR2* locus, a single SNP (rs2501431, A → G) was assessed, which had shown the strongest association in the previously published French sample. BMD, measured by peripheral quantitative computed tomography or dual-energy X-ray absorptiometry, was consistently lower in women with the AA genotype compared to the AG and GG genotypes. Together, these studies strongly suggest that *CNR2* is the susceptibility gene for low BMD and osteoporosis on chromosome 1p36.

Conclusions

Recent studies in mice and humans suggest an important role for the endocannabinoid system in the regulation of skeletal remodeling and the consequent implications on bone mass and biomechanical function. Although the CB1 cannabinoid receptor has been identified in sympathetic terminals innervating the skeleton, its role in controlling bone turn-over remains to be elucidated. The CB2 cannabinoid receptor is expressed in bone cells. Its bone anabolic action, including some of the mechanisms involved, has been reported in some detail, and is also inferred from human genetic studies. These studies portray polymorphisms in *CNR2*, the gene encoding CB2, as important genetic risk factors for osteoporosis. Taken together, the reports on cannabinoid receptors in mice and humans pave the way for the development of 1) diagnostic measures to identify osteoporosis-susceptible polymorphisms in *CNR2*, and 2) cannabinoid drugs to combat osteoporosis.

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