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Article

Mechanisms of Osteoclastogenesis Inhibition by a Novel Class of Biphenyl-Type Cannabinoid CB_2 Receptor Inverse Agonists

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SUMMARY

The cannabinoid CB_2 receptor is known to modulate osteoclast function by poorly understood mechanisms. Here, we report that the natural biphenyl neolignan 4′-*O*-methylhonokiol (MH) is a CB_2 receptor-selective antiosteoclastogenic lead structure (K_i < 50 nM). Intriguingly, MH triggers a simultaneous G_i inverse agonist response and a strong CB_2 receptor-dependent increase in intracellular calcium. The most active inverse agonists from a library of MH derivatives inhibited osteoclastogenesis in RANK ligand-stimulated RAW264.7 cells and primary human macrophages. Moreover, these ligands potently inhibited the osteoclastogenic action of endocannabinoids. Our data show that CB_2 receptor-mediated cAMP formation, but not intracellular calcium, is crucially involved in the regulation of osteoclastogenesis, primarily by inhibiting macrophage chemotaxis and TNF-α expression. MH is an easily accessible CB_2 receptor-selective scaffold that exhibits a novel type of functional heterogeneity.

INTRODUCTION

The cannabinoid type 2 (CB_2) receptor is a G protein-coupled receptor (GPCR) widely expressed in different tissues, including bone (Atwood and Mackie, 2010; Bab and Zimmer, 2008). Modulation of CB_2 receptor activity has been shown to be involved in the pathophysiology of different diseases, including osteoporosis, atherosclerosis, chronic pain, cancer, and distinct inflammatory conditions (Di Marzo, 2009; Buckley, 2008). The CB_2 receptor is part of the endocannabinoid system (ECS), which comprises the formation, transport, and degradation of the endogenous ligands arachidonoyl ethanolamide (anandamide; AEA) and 2-arachidonoyl glycerol (2-AG) (Muccioli, 2010). AEA and 2-AG nonselectively bind to both CB_1 and CB_2 receptors with K_i values in the lower nanomolar range (Hanus and Mechoulam, 2010). The action of endocannabinoids at the CB_2 receptor typically starts the regulation of cAMP formation (via G_i proteins) (Bosier et al., 2010). This GPCR is negatively coupled via $G_{i/o}$ to adenylyl cyclase (AC), and CB_2 inverse agonists (including SR144528) stimulate forskolin-activated AC activity (Rinaldi-Carmona et al., 1998). 2-AG has been shown to be a full agonist of the CB_2 receptor as it induces a maximal inhibitory G_i response leading to full cAMP inhibition and also leads to significant $[Ca^{2+}]_i$ transients in peripheral cells including myeloid cells (Sugiura et al., 2000). The CB_2 receptor-mediated release of intracellular calcium in myeloid cells has been suggested to potentially play a role in immune cell function (Chicca et al., 2009; Gokoh et al., 2007; Sugiura et al., 2000). Independent studies with CB_2 receptor agonists have reported a CB_2 receptor-mediated induction of $[Ca^{2+}]_i$ release in different cells, as well as the modulation of different mitogen-activated protein (MAP) kinases (Shoemaker et al., 2005a; Malysz et al., 2009). However, not all CB_2 receptor agonists trigger a $[Ca^{2+}]_i$ release and not all CB_2 receptor-selective antagonists (inverse agonists) are able to block these $[Ca^{2+}]_i$ transients (Shoemaker et al., 2005b; Sugiura et al., 2000). Thus, the mechanism of CB_2 receptor-mediated induction of $[Ca^{2+}]_i$ and its potential functional effects in primary cells remains poorly understood. Moreover, $[Ca^{2+}]_i$ triggered via CB_2 receptors has only played a marginal role in the classification of CB_2 receptor ligands. Typically, CB_2 receptor agonists, neutral antagonists, inverse agonists and protean agonists are generally described based on their G_i coupled effects (Bosier et al., 2010).

The CB_2 receptor has been independently shown by different groups to regulate osteoclastogenesis and bone resorption and to play a role in bone homeostasis (Karsak et al., 2005; Idris et al., 2005, 2008; Ofek et al., 2006). In addition to expression in osteoclasts, the CB_2 receptor is also expressed in osteoblasts where it was recently shown to stimulate bone formation via a G_i-cyclin D1 mechanism (Ofek et al., 2011). The ECS is most strongly expressed in monocytes/macrophages, which are the precursor cells of osteoclasts (Nakashima and Takayanagi, 2009; Matias et al., 2002). Thus, macrophage differentiation into osteoclasts may be critically modulated by the function of the ECS through

activation of CB receptors. Upon stimulation of monocytes/macrophages by receptor activator of NF-kB ligand (RANKL), the transcription factor NF-ATc is activated and the monocytes/macrophages start to fuse and differentiate to form multinucleated cells (syncytia), the preosteoclasts. Upon activation, preosteoclasts mature and become capable of bone resorption (Negishi-Koga and Takayanagi, 2009). Therefore, osteoclast function is a primary target in the therapy of osteoporosis, which is reflected by the clinical use of potent bisphosphonate osteoclast inhibitors and, more recently, the cathepsin K inhibitors (Stoch and Wagner, 2008). Noteworthy, CB_2 receptor-selective ligands have been shown to be significant modulators of osteoclast function with a potential to treat osteoporosis (Bab, 2007; Idris and Ralston, 2010).

Opinions differ with regard to whether CB_2 receptor agonists or antagonists (inverse agonists) could be exploited to therapeutically target bone degeneration (Lunn et al., 2008). While some researchers concluded that CB_2 receptor activation leads to inhibition of bone formation (for review, see Bab et al., 2009), other researchers argue in the opposite direction (Lunn et al., 2007; Idris and Ralston, 2010). Thus, the data on effects of different CB_2 receptor-selective ligands on osteoclast development and function are conflicting. Given the lack of insight into the CB_2 receptor-mediated effects in these cells, a better understanding of the CB_2 receptor-mediated signal transduction in osteoclastogenesis appears mandatory to shed light on their role in bone formation.

In this study we report the discovery of an easily accessible antiosteoclastogenic biphenyl scaffold derived from the neolignan 4′-*O*-methylhonokiol (MH) naturally occurring in *Magnolia grandiflora* L. seeds. MH binds to CB_2 receptors with high affinity and good selectivity over CB_1 receptors and acts as a CB_2 receptor inverse agonist. Some of the 44 MH derivatives exhibited potent inverse agonist effects and showed a simultaneous $[Ca^{2+}]_i$ stimulation, whereas few derivatives primarily affected $[Ca^{2+}]_i$.

Although $[Ca^{2+}]_i$ regulation is of paramount importance for osteoclastogenesis and bone resorption (Negishi-Koga and Takayanagi, 2009), the involvement of CB_2 receptor-mediated $[Ca^{2+}]_i$ transients in osteoclastogenesis has not been elucidated so far. An increase of $[Ca^{2+}]_i$ would be expected to trigger osteoclast formation via activation of the transcription factor NF-ATc. By using a novel type of CB_2 receptor ligands we have assessed the importance of the CB_2 receptor-mediated cAMP and the $[Ca^{2+}]_i$ modulation for osteoclastogenesis.

RESULTS

Identification and Characterization of CB_2 Receptor-Selective Biphenyl Neolignans in *Magnolia grandiflora* Seed Oil

In a screening initiative aimed at identifying new CB_2 receptor-selective ligands from plant extracts and purified secondary metabolites (Gertsch et al., 2008, 2010), the seed oil of *Magnolia grandiflora* L., a medicinal plant native to the southeastern United States and northern Mexico, scored as the most significant hit out of more than 450 extracts. MH is only a minor secondary metabolite in Asian *Magnolia* species but it is the major constituent of *M. grandiflora* seeds. *M. grandiflora* seeds have been used in traditional Mexican medicine to treat different ailments such as spasms, infertility, epilepsy, and inflammatory diseases (Martinez, 1959; Mellado et al., 1980). An overview of screening data typically obtained in the radioligand displacement assays using the high-affinity cannabinoid receptor ligand [^{3}H]CP55,940 and membrane preparations from human CB_2 receptor-transfected HEK293 cells is shown in Figure 1A. The subsequent isolation of the major secondary metabolites in the *M. grandiflora* seed oil led to the identification of the biphenyl neolignans magnolol, honokiol, and 4′-*O*-methylhonokiol (MH) as active constituents (structures shown in Figure 1B).

The binding affinities of the neolignans to CB receptors were determined in radioligand displacement assays (Figure 1C) using membranes from CB_1 and CB_2 receptor-transfected HEK293 cells. Hill plots and K_i values were generated as previously described (Gertsch, 2008). While the stereoisomers magnolol and honokiol showed moderate CB_2 receptor binding affinity (K_i values ~1–3 μM), the natural honokiol derivative MH with a methoxy group at 4′ exhibited a remarkably strong binding interaction with the human CB_2 receptor (K_i = 43.9 ± 5.2 nM), clearly accounting for most of the positive hit signal detected in the initial screening. In the same assay, AEA, 2-AG and AM630 exhibited K_i values of 278 ± 17, 892 ± 51, and 35.8 ± 9 nM, respectively (Table 1). All three natural biphenyls showed a significantly weaker binding affinity toward the human CB_1 receptor (Figure 1C), the K_i value of MH being 2.4 ± 0.2 μM. Noteworthy, the natural product MH shares structural similarities with some minor constituents reported from *Cannabis sativa* L., i.e., cannabinodiol and cannabinodivarin (Vollner et al., 1969), as well as with the synthetic CB receptor ligands CP55,940 and HU308 (see Figure S1 available online). Since biphenyl containing compounds are potentially privileged structures that may easily undergo nonspecific interactions with proteins (Costantino and Barlocco, 2006), we next profiled magnolol, honokiol and MH in a receptor screen comprising 50 pharmacologically relevant protein targets (carried out at Novartis, Switzerland). The results of this screen are summarized in Figure S2 and indicate a pronounced specificity of MH for the cannabinoid CB_2 receptor.

MH Exerts CB_2 Receptor-Dependent Mixed Inverse Agonist and Agonist Effects

To assess the CB_2 receptor-mediated functional effects of MH (i.e., agonist, inverse agonist/antagonist, and silent antagonist), cellular assays were carried out to measure forskolin-activated and constitutive cAMP. In an assay using CHO-K1 cells stably transfected with *h*CB_2 receptors MH showed significant inverse agonist effects, comparable to those evoked by the potent and commercially available CB_2 receptor-selective inverse agonists AM630 and SR144528 (Figure 2A). To examine whether MH was able to antagonize the G_i effects, i.e., the inhibition of cAMP formation triggered by CB_2 receptor agonists when stimulated with 3 μM forskolin, the high-affinity ligand WIN55212-2 (*h*CB_2 K_i ~3 nM) was incubated alone (500 nM) and in the presence of increasing concentrations of the inverse agonists MH and AM630. In these experiments, the antagonistic effect of MH on the G_i protein-mediated inhibition of cAMP formation by WIN55212-2 was in the range of that of AM630 (Figure 2B).

The CB_2 receptor-selective inverse agonist SR144528 was previously shown to be an effective antagonist of 2-AG-stimulated

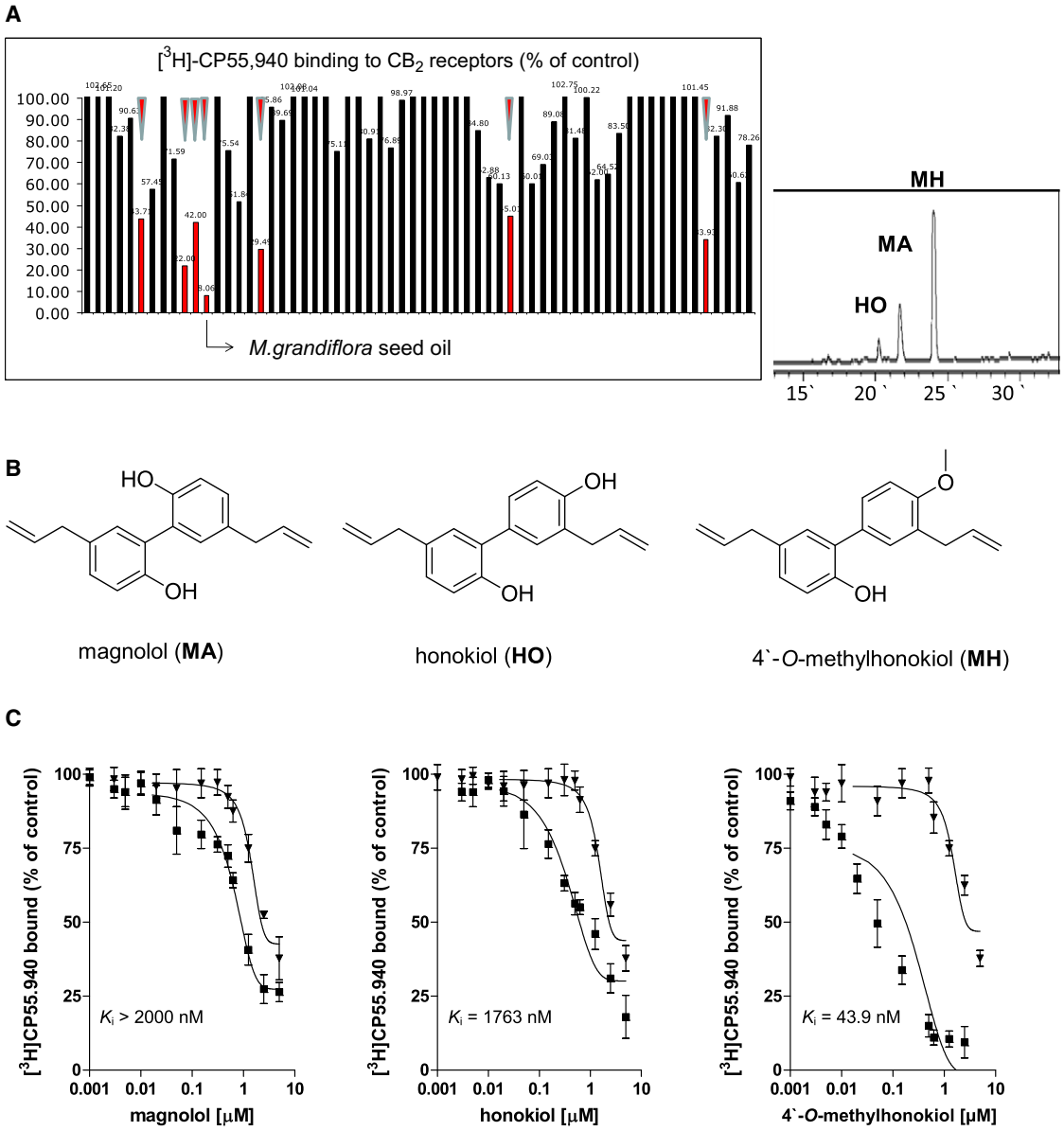

Figure 1. Elucidation of Positive Hit in *M. grandiflora* Seed Oil (10 μg/ml) in Radioligand Displacement Assay Using *h*CB_2 Receptor-Transfected HEK293 Cell Membranes

(A) Shown is a representative test batch of 50 plant extracts. Several of the other positive hits with a displacement >50% (indicated with triangles) were due to the widespread plant natural product β-caryophyllene as assessed by GC/MS dereplication experiments. The *Magnolia* seed oil contains three major neolignan constituents visible in the HPLC chromatogram (on Agilent Zorbax RP-C18, 2.1 × 250 mm columb, flow 300 μl/min, gradient acetonitrile (10%–90%, 30 min) in water, detection Agilent 1100 series diode array detector, temperature controlled at 25°C).

(B) Structures of major secondary metabolites in *M. grandiflora* seed oil.

(C) Concentration-dependent displacement of [^{3}H]CP55,940 in *h*CB_2 (squares) and *h*CB_1 (triangles) receptor-transfected HEK293 cell membranes by magnolol, honokiol, and MH. The biphenyl MH is structurally related to cannabinoids (see also Figure S1) and specifically interacts with CB_2 receptors (see Figures S2 and S3) Determination of K_i values was based on the IC_{50} values from linearized data generated in Hill plots (not shown). Data are mean values ± SD (N = 4).

$[Ca^{2+}]_i$ in myeloid HL60 cells, whereas the inverse agonist AM630 in the same assay was ineffective (Sugiura et al., 2000), indicating that different CB_2 receptor inverse agonists exert distinct effects on 2-AG-triggered $[Ca^{2+}]_i$. We tested MH together with SR144528 and AM630 in an assay measuring inhibition of 2-AG-induced CB_2 receptor-stimulated $[Ca^{2+}]_i$ in an experimental setup previously reported (Raduner et al., 2006). Rather unexpectedly, instead of blocking the effects of 2-AG like SR144528 or being silent like AM630, MH potentiated the $[Ca^{2+}]_i$ induced in HL60 cells by 2-AG (Figure 2C). When incubated alone, MH stimulated $[Ca^{2+}]_i$ in HL60 cells in a concentration-dependent manner (EC_{50} value ~3 μM), with a potency comparable to that of the endocannabinoid agonist 2-AG (Figure 2D) or β-caryophyllene, a natural CB_2 receptor-selective full agonist (Gertsch et al.,

Table 1. Summary of Binding Interactions with *h*CB_1 and *h*CB_2 Receptors

Cpd/Magreth No	CB_2 Receptor K_i Value (nM)	CB_1 Receptor K_i Value (nM)
AM630	35.8 ± 9	>3000
CP55,940	0.83 ± 0.09	0.49 ± 0.06
AEA	278 ± 17	ND
2-AG	892 ± 51	ND
MH	43.9 ± 5	2400 ± 200
1	74.0 ± 5	>2000
2	1305 ± 34	>5000
3a	40.8 ± 12	>5000
3b	59.1 ± 4	>5000
4	1763 ± 83	>5000
11a	1075 ± 14	>5000
11b	84 ± 14	860 ± 63
11c	427 ± 21	>5000
12a	1264 ± 60	>5000
12b	680 ± 49	>5000
14a	138 ± 7	790 ± 21
15	144 ± 5	>3000
16a	51 ± 1	1230 ± 30
16b	40 ± 3	970 ± 19
17a	57 ± 2	>5000
17b	46 ± 4	>5000
18a	102 ± 4	>5000
18b	>3000	>5000
19	134 ± 12	>3000

Shown are the mean K_i values ± SD of at least three independent measurements.

2008). Thus, while acting as a CB_2 receptor inverse agonist at the level of cAMP, rather paradoxically, MH also showed agonist-like effects at the level of $[Ca^{2+}]_i$ in HL60 cells. Because the biphenyl neolignan MH structurally resembles the GPR55 receptor agonists Abn-CBD (abnormal cannabidiol) and O-1602, we investigated the possibility that MH may trigger $[Ca^{2+}]_i$ in HL60 cells via the cannabinoid-like receptor GPR55 (Ross, 2009). In our assay, the GPR55 receptor agonists Abn-CBD and O-1602 led to very weak $[Ca^{2+}]_i$ in HL60 cells which could not be blocked by SR144528 (data not shown). This is in agreement with the observation that SR144528 and 2-AG do not functionally modulate GPR55 receptor activity (Mackie and Stella, 2006). MH did not interact with GPR55 receptors and did not lead to receptor stimulation at low μM concentrations in *h*GPR55 transfected HEK cells (see Figure S3). On the other hand, the $[Ca^{2+}]_i$ transients in HL60 cells triggered by MH could be blocked by SR144528 (Figure 2D), indicating that both compounds share the same or overlapping binding sites despite of their functional differences. The competitive binding to the CP55,940 binding site by MH and SR144528 was substantiated by Dixon analyses (see Figure S4). We also measured $[Ca^{2+}]_i$ in the stably CB_2 receptor-transfected CHO-K1 cell line in which we determined the inverse agonist effects on cAMP (Figure 2F). Although the $[Ca^{2+}]_i$ transients induced by MH were clearly smaller than in HL60 cells, they were reproducible and could be inhibited by SR144528. Moreover, MH did not show an effect on $[Ca^{2+}]_i$ in CB_1 receptor-transfected or untransfected control cells (Figure 2F). Based on our finding that the natural product MH exerts an apparently mixed (heteroactive) action at the CB_2 receptor, leading to both a potent inverse agonist effect at the level of cAMP and a significant agonistic effect at the level of $[Ca^{2+}]_i$, we next explored the underlying structure-activity relationships (SAR) of this biphenyl scaffold in the context of their action on macrophage biology and osteoclast formation.

Synthesis of Neolignan Derivatives and Measurements of CB Receptor Binding Interactions

Given the easy accessibility of its structure, e.g., the lack of stereocenters, a series of derivatizations on MH was carried out in order to explore structural prerequisites for its activity. The respective series of honokiol derivatives were obtained subsequently through demethylation. In addition, de novo syntheses of biphenyls were carried out allowing the functionalization of the aryl positions not easily accessible in naturally occurring neolignans. As shown in Figure 3, the olefinic group of the side chains, the phenolic hydroxyl group, and the aryl positions were modified. The derivatizations based on MH comprised hydrogenation, isomerization, hydroxylation, elongation, and bromination of the side chains (**Magreth-1** to **-12b**). The acronym **Magreth** stands for **Ma**gnolia **Gr**az and **ETH**. Derivatization of the phenolic groups included acetylation and alkylations (**Magreth-13a** to **-18c**). Moreover, in ring A the introduction of an amino or nitro group was carried out leading to derivatives **Magreth-19a** to **-22**. Table S2 shows methylated magnolol derivatives (**23-24b**). In Table S3, biphenyls are shown which were obtained by de novo syntheses (**25-33**) with the exception of commercially obtained **25** and **26**. The syntheses were performed with the intention to obtain as many derivatives as possible using straightforward methodologies. The resulting library of 47 biphenyl-type compounds (see Tables S1–S3) was then subjected to SAR relationship studies comprising cannabinoid receptor binding affinity and functional effects mediated via the CB_2 receptor. The synthetic steps leading to the most potent CB_2 receptor inverse agonists are provided in Figure 3.

The relative binding affinities toward cannabinoid receptors CB_1 and CB_2 are shown in Table 1. Although different biphenyls show significant differences in binding affinity toward the CB_1 receptor, none of the compounds which displaced [H^3]-CP55,940 by more than 50% at 10 μM, yielded a K_i value significantly lower than 1 μM. This contrasts the overall good CB_2 receptor binding affinity of various biphenyls which exhibited K_i values in the lower nM range. In the following, the structure-CB_2 receptor binding relationships are summarized. Tetrahydrohonokiol and tetrahydromagnolol showed a significantly reduced CB_2 receptor binding affinity. Likewise, isomerization of the side chain attached to ring B reduced the binding affinity. Three derivatives here referred to as **Magreth-31** to **33** based on the symmetrical magnolol skeleton were synthesized in order to elucidate the effect of different alkyl side chains. **Magreth-31**, bearing methyl groups instead of allyl rests, completely lost CB_2 receptor affinity. **Magreth-32** and **33** with *tert*-butyl and isobutyl groups, respectively, showed the same CB_2 binding affinity as the parent compound. **Magreth-29**, an isomer of magnolol

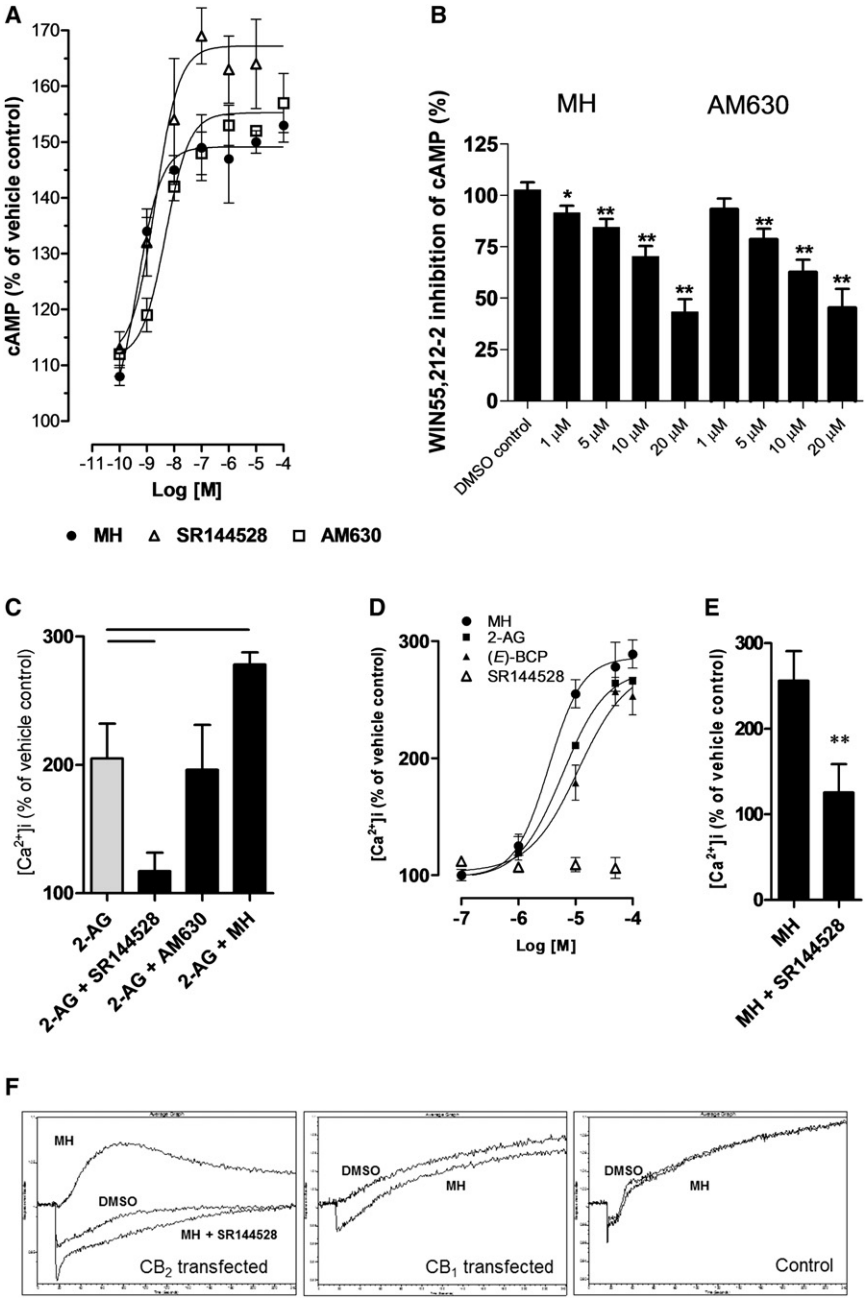

Figure 2. Characterization of Functional Effects of MH

(A) Concentration-dependent inverse agonism (potentiation of forskolin-activated [2 μM] cAMP levels) in confluent *h*CB_2 receptor-transfected CHO-K1 cells by MH, SR144528, and AM630.

(B) MH inhibits the effect of WIN55,212-2 (500 nM) on forskolin-stimulated cAMP in *h*CB_2 receptor-transfected CHO-K1 cells like AM630.

(C) MH potentiates the effect of 2-AG on intracellular calcium ($[Ca^{2+}]_i$) in HL60 cells unlike SR144528, which inhibits this effect, and AM630 which is silent.

(D and E) (D) Concentration-dependent effect of MH on intracellular calcium in HL60 cells, 2-AG, β-caryophyllene (*E*-BCP), and SR144528 were used as controls, and (E) blockage of this effect at 10 μM MH by SR144528 (5 μM). Data are mean values ± SD (N = 3).

(F) Effects of MH (2 μM) on $[Ca^{2+}]_i$ in CB_1 and CB_2 receptor-transfected and untransfected CHO-K1 control cells. SR144528 (10 μM) inhibited the $[Ca^{2+}]_i$ triggered by MH, indicating an overlapping binding site (see also Figure S4). Data were obtained in FLIPR experiments and show mean traces (N = 6). A representative experiment is shown.

bearing the side chains in 3 and 3′ position, turned out to be inactive.

Hydrogenation of the side chain in MH did not significantly change the binding affinity. Bromination of the side chain in subsystem A greatly reduced affinity, whereas bromination in system B did not change the binding affinity observed with MH. As could be seen for **Magreth-12a** and **12b**, the respective honokiol side chain-brominated products showed an overall greatly reduced binding affinity. Extension of side chains in methylhonokiol by an *n*-butyl group led to a dramatic decrease of binding affinity. Epoxidation or hydroxylation of both allyl groups abolished CB_2 receptor binding affinity. Simplified biaryls such as the biphenylols **Magreth-25** and **26** also possessed no binding affinity. Depending on their length, ether-linked alkyl rests at positions 2 and 4′ had a different influence on the CB_2 receptor affinity. For *O*-methyl rests, compounds with ethers in both 2 and 4′ positions showed high binding affinity (**Magreth-14a** and MH); however, the ether linkage with alkyl chains (C_2–C_5) at position 2 showed a more notable effect on binding affinity than alkyl chains attached at 4′. Alkyl chains > 2 carbons at 4′ led to an abolishment of CB_2 affinity (e.g., in **Magreth-17a/b** versus **Magreth-16a/b**). The di-*O*-alkylated compounds in general showed a significant loss of binding affinity (**Magreth-14b**, **16c**, and **18c**). This reduction of binding activity is also observed in a derivative containing hydrophobic groups such as –OAc (**Magreth-13a/b**) or $–OCH_3$ and $–Si(CH_3)_2(t\text{-Bu})$ in **Magreth-9** at positions 2 and 4′. The introduction of a nitro group to position 3 in ring A of MH diminished the CB_2 binding affinity, whereas an amino function at the same position increased binding affinity.

Characterization of the Effects of Magreth Ligands on G_i and $[Ca^{2+}]_i$ Signals

The **Magreth** compounds listed in Table 1 were subjected to functional assays measuring both cAMP in stably *h*CB_2 receptor-transfected CHO-K1 cells and $[Ca^{2+}]_i$ in CB_2 receptor expressing HL60 cells (see Figure S5). In particular, we were interested to see whether the SAR correlated between the inverse agonist (antagonist) and agonist effects originally

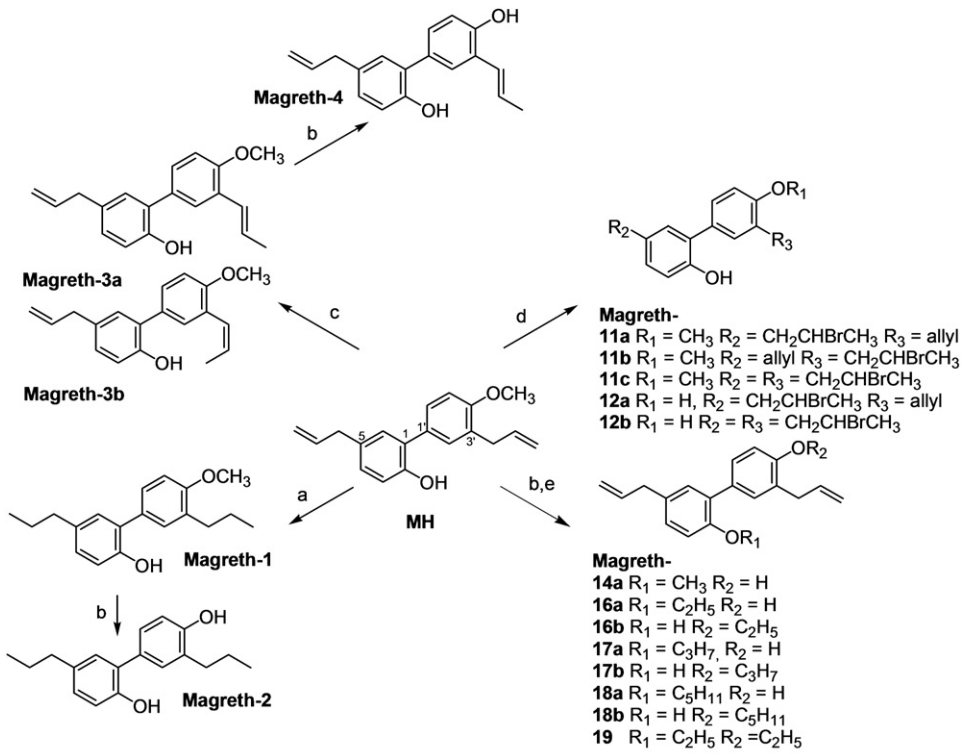

Figure 3. Synthetic Steps Involved in the Generation of the Most Potent CB_2 Receptor-Selective Inverse Agonists
See also Tables S1, S2, and S3.

observed with the natural product MH (vide supra). A strong positive correlation was found between the K_i values of the compounds and their effects on $[Ca^{2+}]_i$, (r^2 = 0.82) but was less pronounced with inverse agonism at the level cAMP (r2 = 0.59). While **Magreth-16b**, **17a**, and **19** showed a more pronounced $[Ca^{2+}]_i$ (agonistic) effect, these compounds did not show inverse agonism and therefore behaved like partial agonists. In all cases, SR144528 blocked the $[Ca^{2+}]_i$ (not shown), thus indicating a CB_2 receptor-mediated mechanism. Generally, the biphenyls more easily activated CB_2 receptor-mediated $[Ca^{2+}]_i$ than G_i effects. **Magreth-3b** was the only compound that showed a more pronounced modulation of cAMP over $[Ca^{2+}]_i$. Because **Magreth-16a** was more potent than MH as CB_2 receptor inverse agonist (cAMP) and agonist ($[Ca^{2+}]_i$), (see Figure S5), this compound was selected for further cellular studies (vide infra).

Effects of Different Biphenyls on Monocyte/Macrophage Chemotaxis and Osteoclastogenesis

Endocannabinoids have been shown previously to induce and/or positively regulate chemotaxis in immune cells, including macrophages (Oka et al., 2004). Along the same line, CB_2 receptor-selective inverse agonists have been shown to inhibit chemotaxis (Lunn et al., 2006). CB_2 receptor-selective inverse agonists have also been shown to inhibit osteoclastogenesis and to protect against bone loss (Lunn et al., 2007). We assessed the effects of the most potent **Magreth** ligands on 2-AG-induced macrophage chemotaxis and MCSF/RANKL-stimulated osteoclast formation. We expected that more potent effects on either cAMP or $[Ca^{2+}]_i$ may lead to more pronounced effects on chemotaxis and/or osteoclastogenesis. As shown in Figure 4, the more potent inverse agonists, including the natural product MH, more potently inhibited 2-AG-induced chemotaxis. **Magreth-16a** (1 μM) and MH potently inhibited both 2-AG-induced chemotaxis and osteoclastogenesis (Figures 4 and 5). The antiosteoclastogenic effects of the **Magreth** compounds were observed at the level of reduced TRAP staining and abolished RANK surface expression in assays in which compounds were coincubated with M-CSF/RANKL-stimulated monocytes/macrophages to initiate osteoclastogenesis (Figure 5). During osteoclastogenesis RANK expression was induced 8- to 15-fold as determined by FACS immunofluorescence. Five hundred nanomolars of MH and **Magreth 1, 3a, 3b, 11b, 16a, and 16b** significantly inhibited osteoclast formation by 50% or more as determined by the inhibition of syncytia formation (only shown for MH and **16b**), the number of tartrate-resistant acid phosphatase-positive (TRAP+) cells, and RANK surface expression (Figure 5). While in experiments using mouse preosteoclasts CB_2 receptor inverse agonist induced apoptosis (Idris et al., 2005), we did not observe apoptotic preosteoclasts or macrophages in our experiments with primary human cells or RAW264.7 cells (data not shown). The potency of the effect on $[Ca^{2+}]_i$ by the **Magreth**

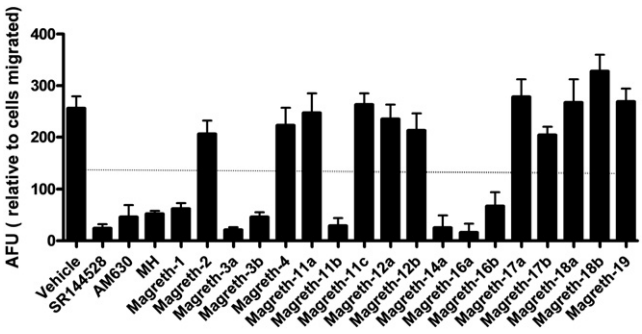

Figure 4. CB_2 Receptor Inverse Agonists Inhibit 2-AG-Induced Macrophage Chemotaxis

Bar graph showing the effect of pretreatment with different CB_2 receptor inverse agonists (10 min, 1 μM at 37°C) on the capacity of primary monocytes/macrophages to migrate toward 2-arachidonoyl glycerol (2-AG) (1 μM) (see also functional effects in Figure S5). Bars below the IC_{50} line were all highly significant. Data are expressed as mean arbitrary fluorescent units (AFU) + SEM (N = 5).

ligands did correlate only weakly with the overall cellular effects (r^2 = 0.49).

Magreth-16a Inhibits the Osteoclastogenic Action of Endocannabinoids

In our assay setup, the endocannabinoids 2-AG and AEA significantly potentiated macrophage colony stimulating factor (MCSF) and RANKL-stimulated osteoclastogenesis in primary monocytes/macrophages at 50–200 nM and thus more physiologically relevant concentrations (Figures 6A–6C). Upon endocannabinoid treatment some osteoclast syncytia were larger than the vehicle (DMSO) treated syncytia, some containing as many as 30 nuclei (Figure 6C). We performed experiments in which both the **Magreth** ligands and the commercial CB_2 receptor-selective inverse agonists SR144528 and AM630 were incubated in the presence of nM concentrations of endocannabinoids. SR144528 and AM630 inhibited the osteoclastogenic effect of AEA and 2-AG (data not shown). As shown in Figure 6B, the osteoclastogenic effect of a mixture of 50 nM of each AEA and 2-AG (replaced every second day) (Figure 6C) was fully inhibited by 200 nM of **Magreth-16a**. Overall, this indicates that the cAMP modulation and not the $[Ca^{2+}]_i$ mediates this action. In our assay setup, the ligands clearly inhibited osteoclast formation beyond the number of osteoclasts formed by endocannabinoid stimulation. The same result was also obtained in RANKL-stimulated RAW264.7 cells (data not shown). This suggested that other osteoclastogenic factors different from endogenous 2-AG and AEA may be modulated via CB_2 receptors. Based on observations with SR144528 reported in an earlier study (Raduner et al., 2006) we speculated that endogenous tumor necrosis factor-α (TNF-α) production may be critically inhibited by the novel CB_2 receptor inverse agonists.

Magreth Derivatives Inhibit Endogenous and LPS-stimulated TNF-α Expression in Monocytes/Macrophages

TNF-α has previously been shown to stimulate osteoclastogenesis and inhibition of this osteoclastogenic cytokine is a widely reported mechanism to inhibit bone resorption (Boyle et al., 2003). Somewhat paradoxically, CB_2 receptor agonists (both full and partial) as well as inverse agonists have been shown to inhibit the expression of proinflammatory cytokines (i.e., TH_1 cytokines) including TNF-α (for review, see Gertsch, 2008). Therefore, the effect of CB_2 receptor ligands on cytokine expression must involve signaling pathways independent of $G_{i/o}$, such as e.g., $[Ca^{2+}]_i$ or kinases. Therefore, we examined the effect of the **Magreth** compounds on the expression of TNF-α in LPS-stimulated primary human monocytes/macrophages and RAW264.7 macrophages. Several of the **Magreth** compounds (500 nM) potently inhibited TNF-α expression from stimulated human monocytes/macrophages (see Figure S6), but they also inhibited the constitutive TNF-α levels from RAW264.7 cells (data not shown). Since the potency of CB_2 receptor interaction correlates with the degree of TNF-α inhibition measured (r^2 = 0.67), a CB_2 receptor-mediated mechanism seems likely, but other mechanisms cannot be excluded. Importantly, the CB_2 receptor-triggered $[Ca^{2+}]_i$ does not seem to play a role in TNF-α inhibition as **Magreth-16b** and **17a** were only moderately effective. Furthermore, in our experimental system SR144528 and AM630 both inhibited TNF-α expression, thus ruling out the possibility of using these pharmacological inhibitors. Endocannabinoids like anandamide also inhibit TNF-α expression (Raduner et al., 2006) and may thus also have antiosteoclastogenic effects, depending on the actual concentration of TNF-α in cell culture media. Five hundred nanomolars of 2-AG inhibited TNF-α by approximately 30% in this assay (data not shown).

DISCUSSION

The natural product 4′-*O*-methylhonokiol (MH) was identified from *Magnolia grandiflora* L. seed oil as a new CB_2 receptor-selective ligand. MH exhibits an intriguing nonspecific heteroactive behavior at the CB_2 receptor, i.e., acting as inverse agonist at $G_{i/o}$ and full agonist at $[Ca^{2+}]_i$, thus providing a novel type of functionally nonspecific CB_2 receptor ligand. This suggests that CB_2 receptors respond to functionally diverse ligands by displaying distinct conformational states with unique kinetic profiles, rather than by acting as simple switches. Alternatively, different subpopulations within the same assay couple to different G proteins and our ligands are able to differentially but simultaneously modulate these. This will have to be addressed in future studies. In the present study, the pharmacophore of MH was investigated by derivatization and the prepared nanomolar CB_2 receptor ligands were used to study effects on osteoclastogenesis. Our results show that the endocannabinoids AEA and 2-AG exert osteoclastogenic effects at low nM concentrations in human preosteoclasts by triggering syncytium formation, and that CB_2 receptor inverse agonists can block this effect via inhibition of 2-AG-triggered chemotaxis. Although 2-AG exhibits a CB_2 receptor K_i value in the low micromolar range (Table 1) this endocannabinoid may activate the CB_2 receptor at significantly lower concentrations without the need of full receptor occupancy. The osteoclastogenic effect of endocannabinoids has been reported previously (Idris et al., 2005) and suggested to be CB receptor dependent, as indicated by the fact that both CB_1 and CB_2 inverse agonists were able to

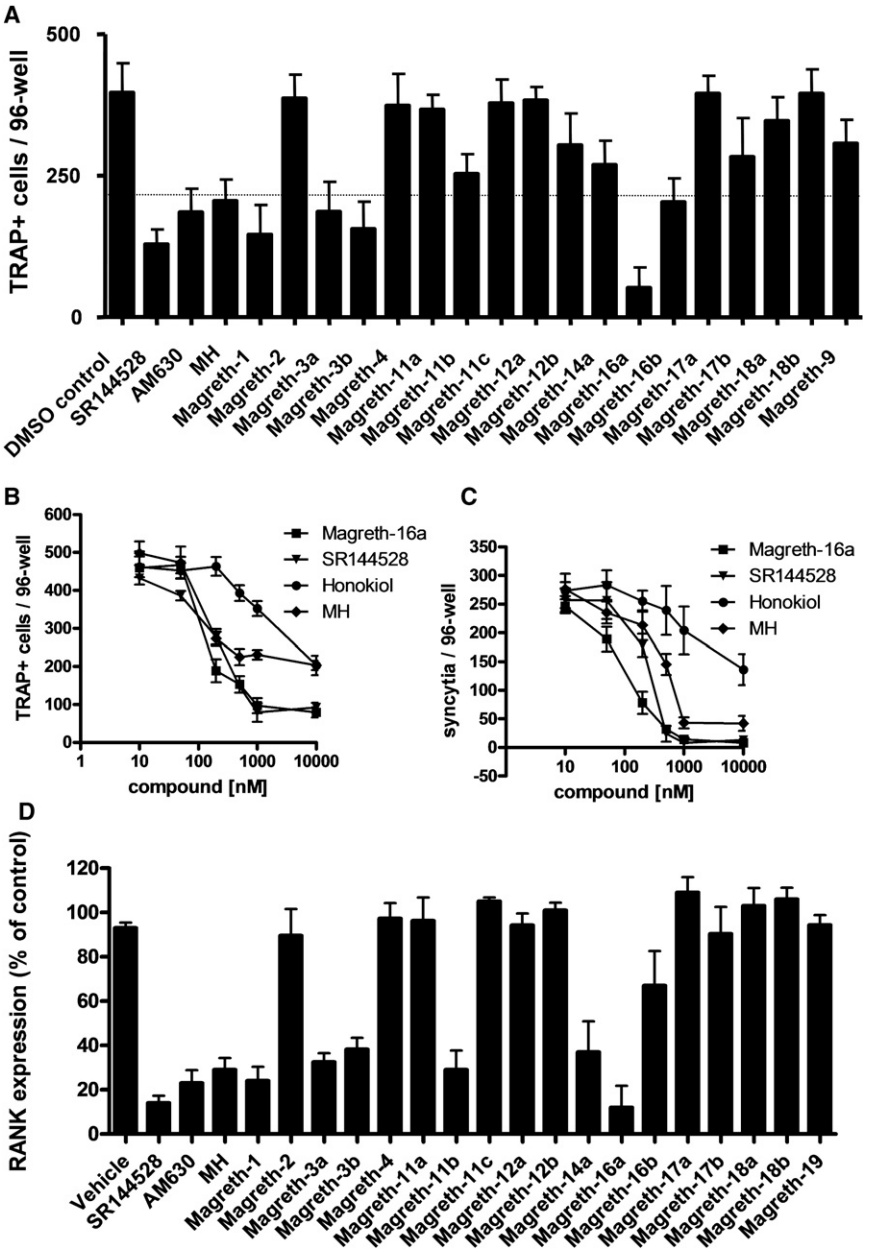

Figure 5. Biphenyl-Type CB_2 Receptor Ligands Inhibit Osteoclastogenesis in Primary Human CD^{14+} Monocytes/Macrophages and RAW264.7 Cells

(A) Effect of **Magreth** compounds (500 nM) on osteoclastogenesis in MCSF (20 ng/ml)/RANKL (50 ng/ml)-stimulated primary CD14+ human monocytes/macrophages as determined by the number of TRAP+ cells. For effects on TNF-α expression see Figure S6. Data show mean values + SD (N = 3) of osteoclasts/96 well at day 14. Medium was changed every second day.

(B) Dose-response curve of inhibitory action of **Magreth-16a**, SR144528, honokiol, and MH in RANKL (100 ng/ml)-stimulated RAW264.7 cells.

(C) Effect of **Magreth-16a,** SR144528, honokiol, and MH on syncytium formation during osteoclastogenesis in RANKL (100 ng/ml)-stimulated RAW264.7 cells. Data show mean values ± SD of at least three independent experiments.

(D) Effect of **Magreth** compounds (500 nM) on osteoclastogenesis in MCSF (20 ng/ml)/RANKL (50 ng/ml)-stimulated primary CD14+ human monocytes/macrophages as determined by RANK surface expression.

block the action of AEA (for review, see Idris and Ralston, 2010). In the case of AEA, the osteoclastogenic effect also involves the TRPV1 channel (Rossi et al., 2009; Idris et al., 2010a) and possibly GPR55 (Whyte et al., 2009). Our data obtained by making use of the biphenyl scaffold substantiate that the classical CB_2 receptor inverse agonists (antagonists) SR144528 and AM630 inhibit osteoclastogenesis independent of $[Ca^{2+}]_i$ modulation but are dependent on cAMP-mediated chemotaxis of preosteoclasts and possibly inhibition of TNF-α expression. Our data further indicate that even though in myeloid cells CB_2 receptor-triggered $[Ca^{2+}]_i$ may be physiologically relevant, this does not seem to play a key role in osteoclast biology. This is corroborated by the finding that both 2-AG and AEA similarly stimulate osteoclastogenesis at low nanomolar concentrations, despite the fact that only 2-AG triggers $[Ca^{2+}]_i$ in myeloid cells. We thus speculate that the CB_2 receptor may switch its functional signaling repertoire upon syncytium formation. On the other hand, a clear correlation was found between the degree of inverse agonism, i.e., cAMP modulation via the CB_2 receptor and the antiosteoclastogenic effects (r^2 = 0.78). TNF-α which is differentially expressed by monocytes/macrophages, also affects osteoclast formation and exerts both osteoclastogenic and antiosteoclastogenic effects, depending on the concentration and assay setup (Balga et al., 2006). TNF-α inhibition is typically also seen with CB_2 receptor agonists (Gertsch, 2008) and this may account for differences in experimental results in osteoclast assays, which may have been generated under different constitutive TNF-α levels and distinct concentrations of CB_2 receptor ligands tested.

Biphenyls are relatively rare in nature and it is quite remarkable that MH is structurally albeit not biosynthetically related to the cannabinodiol-type compounds from *Cannabis sativa* L. that are derived from cannabinol. Cannabinodiol and cannabinodivarin are the only known *Cannabis* biphenyls (see Figure S1) and are only found in trace amounts (Vollner et al., 1969; Turner et al., 1980). To our knowledge, they have never been tested for their action on CB receptors. A synthetic CB_2 receptor-selective agonist with certain structural similarity to MH is HU308, which has been shown to inhibit osteoclastogenesis (Ofek et al., 2006). In an in vitro screen of a panel of 50 receptors, MH only showed ligand binding interactions with CB receptors (see

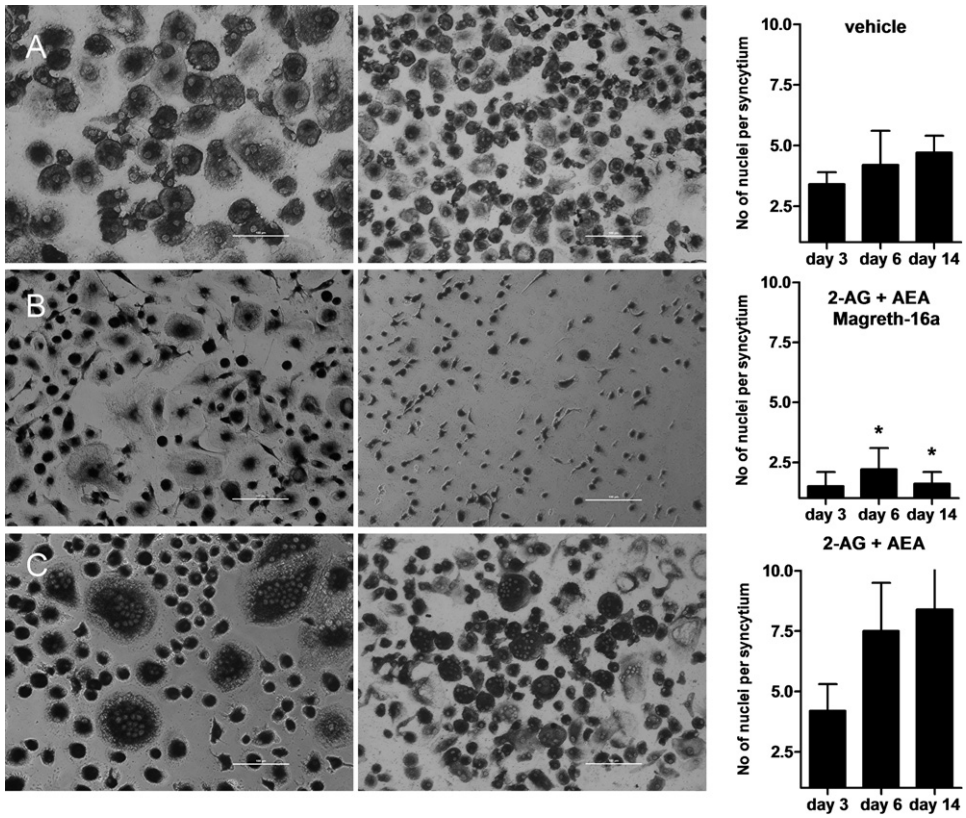

Figure 6. Magreth-16a Inhibits Osteoclastogenesis of MCSF (20 ng/ml)/RANKL (50 ng/ml)-Stimulated Primary Human CD14+ Monocytes/Macrophages

Stimulation/treatment every second day over 14 days. Shown are TRAP+ stained cells (dark color), size bar is 100 μm, left 20× magnification, right 10× magnification (A) Vehicle control, (B) Antiosteoclastogenic effect of **Magreth-16a** (200 nM) in the presence of a combination of 50 nM of each 2-AG and AEA, (C) Effect of a combination of 50 nM of each 2-AG and AEA. Data show mean values + SD (N = 3). Paired *t* test *, $P<0.05$.

Figure S2) but did not show apparent effects on GPR55 (see Figure S3). MH may therefore be highly selective toward CB_2 receptors. Nevertheless, MH has previously been shown to also inhibit COX-2 in vitro (Schühly et al., 2009). Interestingly, structurally related biphenylcarboxylic acid derivatives have been described as a novel and therapeutically promising class of bone resorption inhibitors (Van't Hof et al., 2004; Idris et al., 2010b) and could theoretically exert their antiresorptive effects via CB_2 receptor interactions. Honokiol, which binds to CB_2 receptors at low μM affinity (Figure 1), has been shown previously to inhibit osteoclastogenesis in vitro at μM concentrations (Ahn et al., 2006; Hasegawa et al., 2010), but in these studies no reference was made to the ECS. The heteroactive (functionally nonspecific) **Magreth** compounds reported here represent a novel class of CB_2 receptor ligands. They may be explored in physiological systems and in disease contexts like bone loss.

SIGNIFICANCE

The CB_2 receptor has attracted attention as a validated target for the development of new therapeutic strategies (Buckley, 2008) with an array of functionally different cannabinoid receptor ligands already reported (Pertwee et al., 2010). Our finding of a functionally novel class of a CB_2 receptor heterocative molecular template, which exerts mixed inverse agonist responses together with a simultaneous CB_2 receptor-mediated $[Ca^{2+}]i$ either reflects the pharmacophore complexity of the CB_2 receptor or an unexpected heterogeneity of CB_2 receptor coupling within the same cell line. The first would indicate that the current concept of a bifunctional switch may be insufficient, thus adding a new level of complexity to CB_2 receptor pharmacology.

The inhibition of 2-AG-triggered chemotaxis of pro-osteoclasts and inhibition of syncytium formation by CB_2 receptor inverse agonists (via cAMP) may help to further elucidate the role of CB_2 receptors in bone homeostasis. Osteoclasts are cells of hematopoietic origin with a functional endocannabinoid system and the unique property of dissolving bone; their inhibition is a hallmark for the treatment of diseases of bone loss.

The biphenyl neolignan methylhonokiol, isolated from the medicinal plant *Magnolia grandiflora*, is an easily accessible and apparently nontoxic CB_2 receptor ligand with potential for the development of therapeutics for diseases in which the CB_2 receptor may play a role. These include osteoarthritis, neuroinflammation, pain, and chronic bowel disease.

EXPERIMENTAL PROCEDURES

Materials

Sources are given in the Supplemental Information available online.

Data Analysis

Results are expressed as mean values ± SD or ± SEM for each examined group. Statistical significance of differences between groups was determined by the Student's t test (paired t test) with GraphPad Prism5 software. Outliners in a series of identical experiments were determined by Grubb's test (ESD method) with alpha set to 0.05. Statistical differences between treated and vehicle control groups were determined by Student's t test for dependent samples. Differences between the analyzed samples were considered as significant at $p \leq 0.05$. Nonlinear regression analysis (curve fitting) was performed with GraphPad Prism5 software.

Screening of Plant Extract Libraries and Determination of K_i Values and Receptor Screen

The radioligand assays used were described previously (Gertsch et al., 2008). Data were fitted in a sigmoidal curve and graphically linearized by projecting Hill plots, which for both cases allowed the calculation of IC_{50} values. Derived from the dissociation constant (K_D) of [^{3}H]CP-55,940 (0.39 nM) and the concentration-dependent displacement (IC_{50} value), inhibition constants (K_i) of competitor compounds were calculated by using the Cheng-Prusoff equation [$K_i = IC_{50}/(1 + L/K_D)$]. The procedure for the receptor screen is provided in the Supplemental Experimental Procedures.

Cell Cultures

Human promyelocytic leukemia CB_2-expressing (positive) HL60 cells (obtained from the ATCC, CCL-240) were grown in Iscove's modified Dulbecco's medium with 4 mm L-glutamine and 1.5 g/liter sodium bicarbonate (ATCC, Manassas, VA) supplemented with 20% fetal bovine serum, 1 g/ml fungizone (amphotericin B), 100 units/ml penicillin G, and 100 g/ml streptomycin. The human CB_2-expressing CHO-K1 cells were grown in the same medium as the CB_2-negative HL60 cells but supplemented with 400 μg/ml G418 (10131-027; Invitrogen). All cells were grown in a humidified incubator at 37°C and 5% CO_2. Isolation and culture of primary monocytes/macrophages are described in the Supplemental Experimental Procedures.

Monocyte/Macrophage Chemotaxis In Vitro Assay

Primary human monocytes from healthy human donors were obtained as previously reported (Raduner et al., 2006). The monocytes were then differentiated into macrophages in cultured in RPMI 640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin (Invitrogen, Switzerland) in a 5% CO_2-humidified incubator at 37°C. Macrophage cell number was assessed by trypan blue dye exclusion using a hematocytometer. Cells were then collected and prepared as per manufacturer instructions for the QCM™ chemotaxis (8 μM) cell migration assay (Millipore). In brief, 30,000 monocytes/macrophages were seeded in the upper chamber of the provided 96-well plates. The lower chambers were filled with conditioned medium with 2-AG (1 μM) or vehicle control. The plates were then incubated overnight to allow for cell migration through the pores and into the lower chamber or to the outside bottom of the chamber. Any cells attached to the outside of the chamber were detached using the provided detachment buffer and collected according to manufacturer instructions. Migrated cells were detached, lysed, and labeled with a fluorescent dye that exhibits strong fluorescence when bound to cellular nucleic acids. Sample fluorescence was measured with a fluorescence microplate reader (Farcyte Tecan, Switzerland). The excitation maximum was 480 nm; the emission maximum was 520 nm.

Osteoclastogenesis Assays

The assays used to induce osteoclastogenesis in RAW264.7 macrophages and primary human monocytes/macrophages are described in Supplemental Experimental Procedures. The latter was adopted from Susa et al. (2004). In brief, mononuclear cells were isolated from human peripheral blood by density centrifugation, seeded at 600,000 cells per 96-well and cultured for 17 days in α-MEM medium, supplemented with 10% of selected fetal calf serum, 1 μM dexamethasone and a mix of macrophage-colony stimulating factor (M-CSF, 20 ng/ml), receptor activator of NFκB ligand (RANKL, 50 ng/ml), and transforming growth factor-β1 (TGF-β1, 5 ng/ml).

Stimulation of TNF-α in Monocytes/Macrophages

Measurement of TNF-α was carried out as reported previously (Raduner et al., 2006). A detailed procedure is provided in the Supplemental Information available online.

Measurements of $[Ca^{2+}]_i$ Transients in HL60 cells and CHO-K1 cells

Measurements were carried out as reported previously (Raduner et al., 2006). A detailed procedure is provided in the Supplemental Experimental Procedures.

Characterization of Compounds

Characterization is provided in the Supplemental Information.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, Supplemental Experimental Procedures, and three tables and can be found with this article online at doi:10.1016/j.chembiol.2011.05.012.

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