The Natural Product Magnolol as a Lead Structure for the Development of Potent Cannabinoid Receptor Agonists

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Abstract
Magnolol (4-allyl-2-(5-allyl-2-hydroxyphenyl)phenol), the main bioactive constituent of the medicinal plant Magnolia officinalis, and its main metabolite tetraydroxymagnolol were recently found to activate cannabinoid (CB) receptors. We now investigated the structure-activity relationships of (tetrahydro)magnolol analogs with variations of the alkyl chains and the phenolic groups and could considerably improve potency. Among the most potent compounds were the dual CB1/CB2 full agonist 2-(2-methoxy-5-propyl-phenyl)-4-hexylphenol (61a, IC50 = 0.00957 μM; Kᵢ CB1 = 0.0238 μM), and the CB2-selective partial agonist 2-(2-hydroxy-5-propylphenyl)-4-pentyphenol (60, Kᵢ CB1 = 0.362 μM; Kᵢ CB2 = 0.0371 μM), which showed high selectivity versus GPR18 and GPR55. Compound 61b, an isomer of 61a, was the most potent GPR55 antagonist with an IC50 value of 3.25 μM but was non-selective. The relatively simple structures, which possess no stereocenters, are easily accessible in a four- to five-step synthetic procedure from common starting materials. The central reaction step is the well-elaborated Suzuki-Miyaura cross-coupling reaction, which is suitable for a combinatorial chemistry approach. The scaffold is versatile and may be fine-tuned to obtain a broad range of receptor affinities, selectivities and efficacies.

Introduction
Cannabinoid (CB) receptors comprise a small family of G protein-coupled receptors (GPCR) consisting of two subtypes, CB1 and CB2. Both receptors exhibit 44% identity in amino acid sequence, are coupled to G12/13 proteins [1,2], and differ in their expression patterns. The CB1 receptor is predominantly expressed in cells of the central nervous system, however, the CB1 receptor is also found in peripheral tissues like adrenal gland, bone marrow, heart, lung, prostate, testis, tonsils spleen and in adipocytes [1,3]. The CB2 receptor is mainly expressed on cells of the immune system, for example in tonsils and spleen [4,5] but it is expressed in the central nervous system as well [6]. CB receptors are activated by constituents of the plant Cannabis sativa, e.g., Δ⁹-tetrahydrocannabinol (Δ⁹-THC, 1) to which they owe their name. Structures of selected CB receptor ligands are shown in Figure 1. Physiological agonists, the so-called endocannabinoids, include anandamide (2) and 2-arachidonoylglycerol [3] [7].

The orphan receptor GPR55 had been proposed as a third member of the CB receptor family because of its responsiveness to some CB receptor ligands, but several studies suggested that the non-cannabinoid lysophosphatidylglycerol (LPG) may be its physiological agonist [8,9,10]. GPR55 is expressed on cells of the central nervous system, on blood and vascular endothelial cells, bone cells, cells of the immune system and on adipocytes; it is phylogenetically distinct from both CB receptor subtypes, showing a low amino acid identity (CB1 15%, CB2 13%) [11].

Recently, it was discovered that several cannabinoïds, including Δ⁹-THC (1), anandamide (2), and its metabolite N-arachidonoylglycerine were agonists at the orphan receptor GPR18, which makes this receptor a likely candidate for the elusive third CB receptor subtype, despite its very low amino acid identity with the classical cannabinoid receptors (CB1 12%, CB2 7%) [12,13]. The GPR18 is predominantly expressed on cells of testes and spleen, but also on thymus cells, leukocytes, in thyroid, small intestine and stomach [14,15]. Neither potent nor selective ligands for GPR18 have been described so far [16]. Only very few moderately potent and selective ligands for GPR55 have been identified [17,18]. In contrast, several classes of synthetic compounds have been developed that either activate CB receptors, e.g. the nonselective CB1/CB2 agonist CP55,940 (4), or inhibit them, e.g. the CB1-selective inverse agonists rimonabant (5), LY320135 (6), and the CB2-selective inverse agonist AM630 (7) [1,19,20]. Compounds with distinct functional properties at CB receptor subtypes have also been developed, such as PSB-SB-1201 (8), an antagonist at CB1 and an agonist at CB2 receptors [21]. There is widespread potential for therapeutic applications of CB receptor ligands. CB1 receptor agonists, for example, relieve pain, nausea and vomiting, reduce hyperexcitability in epilepsy, and increase food intake of debilitated patients. On the other hand CB1 receptor antagonists may be useful for the modulation of behavior in addiction and for treating obesity [22]. In fact, rimonabant (5) had been approved for that indication, but has meanwhile been withdrawn from the market due to serious side-effects resulting in an increased suicide

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Antinociceptive effects, especially in neuropathic and chronic pain, indications of medical need, may be achieved by selective CB2 receptor agonism thus avoiding adverse CB1 effects [24]. Further fields of application for CB2 receptor agonists are inflammatory diseases including multiple sclerosis and arthritis [25]. A few CB receptor agonists have already been licensed for clinical use, including dronabinol (Δ9-THC), nabilon, a synthetic THC analog, and a combination of Δ9-THC with cannabidiol to reduce psychotropic effects [24].

Since CB receptors are validated drug targets the development of novel, improved ligands is warranted. A large number of therapeutically used drugs are compounds from nature or derivatives thereof [26]. These natural products are drug-like molecules with regard to their physicochemical properties and their ability to interact with biological structures, especially with proteins, since they have been optimized throughout evolution to interact with target structures [26,27].

Natural products have a long history of interactions with CB receptors. Preparations of the medicinal plant Cannabis sativa have been therapeutically used for thousands of years before their mechanism of action – the activation of CB receptors – had been discovered and the active constituents like THC (1) had been identified [28]. In addition to cannabis constituents further plant-derived natural products have been reported to interact with the endocannabinoid system, including the terpene beta-caryophyllene, fatty acid derivatives, such as N-linoleoyethanolamide, and various N-alkylamides from Echinacea spp. [29,30,31]. These compounds may either act directly on CB receptors (beta-caryophyllene) [29] or indirectly by inhibition of endocannabinoide degradation (N-linoleoyethanolamide and various N-alkylamides) [30,31]. Recently we discovered that a bark extract of Magnolia officinalis, which has been used in traditional Chinese medicine (TCM) for the treatment of insomnia, anxiety disorders and allergic diseases [32,33], exhibits CB-agonistic effects [34]. The main active constituents of Magnolia officinalis bark were shown to be the biphenylic neolignans magnolol (9), honokiol (10), and to a very minor content 4'-O-methylhonokiol (11) [33]. It was shown that these biphenylic compounds interact with CB receptors [34,35]. 4'-O-Methylhonokiol (11), the main constituent of the seeds of Magnolia grandiflora L., but only a minor constituent of Magnolia officinalis, was found to be an inverse agonist at CB2 receptors with selectivity versus CB1 (Ks, CB1 2400 nM, Ks, CB2 43.9 nM) [35]. Extensive semisynthetic modification of 11 did not lead to any significant increase in CB2 receptor affinity of the natural product [35].

Figure 1. Structures of selected cannabinoid receptor ligands.
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We recently discovered that the symmetrical biphenol magnolol (9) is a partial agonist at both CB receptor subtypes with $K_I$ values in the low micromolar range, while its main metabolite tetrahydromagnolol (12) possessed 20-fold higher potency at the CB$_2$ receptor along with selectivity for that subtype [34]. In the present study we therefore utilized magnolol (9) and its hydrogenated metabolite 12 as new lead structures for the development of potent and selective CB receptor ligands.

**Results and Discussion**

**Structural Comparison**

Comparison of the structural features of the newly discovered partial CB$_2$ receptor agonist tetrahydromagnolol (12) with those of the natural cannabinoid $\Delta^9$-THC (1), a partial CB$_1$/CB$_2$ agonist, and the synthetic CB$_1$/CB$_2$ full agonist CP 55,940 (4) showed structural similarities (Figure 2). All three structures share two directly connected six-membered rings at least one of which bears a hydroxyl group and an alkyl residue of different chain length. An additional oxygen atom – a hydrogen bond acceptor – is found in a certain distance of the hydroxyl function: in 4 an alkylating agent in a mixture of water and dichloromethane in the presence of sodium hydroxide and benzyl-triethylammonium bromide led to the 2-bromo-1-methoxy-3-alkylbenzenes 30–32 [37]. Boronic acid derivatives 33–38 were obtained from the 2-bromo-4-alkylphenols 21–29 by treatment with n-butyllithium and trimethyl borate (Figure S3) yielding 33–38 after acidic hydrolysis in moderate yields [38]. 2-Methoxy-5-propylphenylboronic acid (39) was obtained by the same procedure.

**Syntheses of Magnolol Derivatives and Analogs**

The final analogs 12, 12a, 40–65, 61a and 61b (Figure 4), were synthesized by Suzuki cross coupling reactions [39]. We found that the coupling resulted in higher yields (ca. 65% compared to ca. 30%) when the 2-bromo-1-methoxy-4-alkylbenzenes 30–32 were used instead of the unmethylated 2-bromo-4-alkylphenols 21–29. Demethylation [40] of 12a, 60a, and 61a yielded the products 12, 60, and 61 (Figure S5). Overall yields when using the methylated phenols 30–32, involving an additional demethylation step, were higher compared to the direct coupling of the 2-bromo-4-alkylphenols (ca. 50% compared to ca. 30%). The final compounds were purified by flash chromatography or HPLC, respectively. The structures were confirmed by $^1$H- and $^{13}$C-NMR spectra and by HPLC coupled to electrospray ionization mass spectrometry (LC-ESI-MS). The purity was confirmed by the same method and was in all cases greater than >95% (for details see Experimental Section and Supporting Information).

**Biological Evaluation**

The affinities of the magnolol analogs at human CB$_1$ and CB$_2$ receptors were determined in radioligand binding studies using $[^3]$H-$\Delta^9$-THC and $[^3]$H-CP55,940 as a non-selective CB receptor radioligand. Membrane preparations of Chinese hamster ovary (CHO) cells stably expressing the respective receptor subtype were utilized as a source for human CB$_1$ or CB$_2$ receptors, respectively. Compounds were initially screened at a concentration of 10 $\mu$M. In cases where inhibition of radioligand binding was about 50% or more, full concentration-inhibition curves were determined in order to calculate $K_I$ values. To

![Figure 2. Structural comparison of $\Delta^9$-THC (1), the synthetic CP55,940 (4), tetrahydromagnolol (12).](https://doi.org/10.1371/journal.pone.0077739.g002)
Investigate the intrinsic activities of the synthesized compounds at CB receptors cAMP accumulation assays with CHO cells stably expressing the human CB1 or CB2 receptor subtype were performed. Intracellular cAMP levels were measured by a radioactive filtration assay determining competition of [3H]cAMP by formed cAMP to a binding protein isolated from bovine adrenal glands [21,41]. Effects of test compounds (10 μM) on forskolin-stimulated cAMP levels were determined relative to the maximal effect observed with the full agonist CP55,940. To determine interaction with the CB-related orphan receptors GPR18 and GPR55 β-arrestin assays with CHO cells stably expressing the respective receptor were carried out. A β-galactosidase enzyme fragment complementation technology was applied (β-arrestin PathHunter™ assay, DiscoverX, Fremont, CA).

**Figure 3. Synthesis of intermediates.** (a) Br2, NaHCO3, CHCl3, 0°C; (b) three steps, (1) n-butyllithium, Et2O, −78°C; (2) B(OCH3)3, Et2O, −78°C to rt; (3) HCl, Et2O; (c) CH3I, NaOH, benzyl-tri-n-butylammonium bromide, CH2Cl2 : H2O (1: 1), 12 h, rt.

**Figure 4. Synthesis of magnolol derivatives and analogs.** (a) Pd[PPPh3]4, Na2CO3, toluene, EtOH, H2O, 100°C, 18h; (b) CH2Cl2, BBr3, −78°C to rt.
CA, USA) to monitor β-arrestin recruitment to activated receptors. The compounds were screened for agonistic and antagonistic activity at the respective receptor at a concentration of 10 μM. Full concentration-effect curves were determined for the most potent compounds. Biological data are collected in Table 1, Table 2 and Table S1. For comparison the results of commercially available standard compounds obtained under the same assay conditions are included.

Structure-Activity Relationships at CB1 and CB2 Receptors

The natural product magnolol (9, 4-allyl-2-(5-allyl-2-hydroxy-phenyl)phenol) was recently found to show affinity for CB1 and CB2 receptors in the low micromolar range behaving as a partial agonist at both receptor subtypes [34,35]. Its main metabolite tetrahydromagnolol (12), which contains two propyl instead of allyl residues due to reductive metabolization, was even more potent and showed selectivity for CB2 receptors [34]. Based on the (tetrahydro)magnolol (9,12) scaffold we replaced the allyl (9) or propyl (12) moieties in the para-position of the phenolic hydroxyl groups by a large variety of different residues ranging from hydrogen to long aliphatic alkyl chains (up to octyl). As a second modification we studied the effect of methylation of one of the phenolic hydroxyl groups in selected derivatives.

As a first step we investigated symmetrically substituted biphenyls. The simplest symmetric biphenyl derivative 2-(2-hydroxyphenyl)phenol (40) displayed no affinity towards CB

| Table 1. Potencies and Efficacies of Magnolol Derivatives and Analogs at human Cannabinoid Receptor Subtypes. a |
| --- |
| **Compd.** | **heterologous competition vs. [3H]CP55,940** | **cAMP accumulation assay** |
| | $K_i$ (nM) | CB1 | CB2 | $EC_{50}$ (nM)/efficacy | CB1 | CB2 |
| 1 | 3.88±0.91 | 71.6±0.024.1 | 6.76±3.61/88% b | 14.0±6.8/34% b |
| 4 | 1.28 [21] | 1.42 [21] | 2.28 [21]/100% b | 1.00 [21]/100% b |
| 9 | 3150 [34] | 1440 [34] | 18300/62% [34] | 3280/31% [34] |
| 10 | 6460 [34] | 5610 [34] | 4% b | 0% b |
| 11 | 8340±3200 (2400 [35]) | 43.3±17.1 (43.9 [35]) | 42% b | 87% b |
| 12 | 2260 [34] | 416 [34] | 9010/124% [34] | 170/49% [34] |
| 12a | 267±58 | 221±57 | 622±284/112% b | 77.8±20.5/83% b |
| 40 | >1000 | >1000 | n.d. | n.d. |
| 41 | >1000 | >1000 | n.d. | n.d. |
| 42 | 2130±840 | 2870±770 | n.d. | (~ 3%) |
| 43 | 2700±1200 | 1590±40 | 7110±1430/100% b | 378±148/67% b |
| 44 | 3130±1130 | 833±123 | 4540±830/44% b | 2300±710/62% b |
| 45 | 4640±580 | 1830±190 | 0% b | 74% b |
| 46 | ~1000 | ~1000 | n.d. | ~1000 |
| 47 | ~1000 | 2030±880 | 0% b | 31% b |
| 48 | 6590±2560 | 1160±290 | 50% b | 51% b |
| 49 | 6630±5030 | 1500±640 | 74% b | 34% b |
| 50 | >1000 | 7380±2760 | n.d. | 5% b |
| 51 | ~1000 | 1690±530 | 0% b | 37% b |
| 52 | 1230±470 | 517±101 | 0% b | 37% b |
| 53 | 822±224 | 273±96 | 105% b | 42% b |
| 54 | ~1000 | 856±367 | 0% b | 12% b |
| 55 | 5760±2850 | 235±101 | 0% b | 30% b |
| 56 | 634±297 | 161±33 | 91% b | 91% b |
| 57 | 386±29 | 83.0±11.8 | 114% b | 47% b |
| 58 | 3610±170 | 468±133 | 79% b | 64% b |
| 59 | 5810±2670 | 489±49 | 36% b | 36% b |
| 60 | 362±113 | 37.1±7.8 | 971±89/98% b | 258±13/81% b |
| 60a | 173±1.4 | 310±9.9 | 37.5±5.6/95% b | 39.9±10.0/94% b |
| 61 | 145±48 | 29.4±9.0 | 829±278/102% b | 159±18/70% b |
| 61a | 9.57±5.43 | 23.8±7.1 | 159±76/100% b | 38.5±17/100% b |
| 61b | 313±125 | 281±101 | $K_i: 1850±730/0% b$ | 595±150/42% b |

a all data resulted from three independent experiments, performed in duplicates.
b efficacy at 10 μM compared to max. effect of the full agonist CP55,940 (1 μM) = 100%.

c efficacy was determined at a concentration of 100 μM.
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receptors. The introduction of alkyl substituents in the R1 and R2 position markedly enhanced CB receptor affinity, with an optimum being reached by propyl substitution (12, Ki CB1:2.26 μM, CB2:0.416 μM). Longer chains (butyl (44), hexyl (45)) resulted in decreased affinities compared to tetrahydromagnolol (12), emphasizing that the di-propyl substituent, both in the R1 and R2 position (also see Table S1). By keeping the residue in the R2-position constant we investigated the influence of the size of the substituent on the CB2 receptor affinity. The introduction of alkyl substituents in the R1 and R2 position (also see Table S1). Due to the symmetrical structure of the biphenol core, the designation R1 and R2 is interchangeable; only in compounds where one of the phenolic groups is alkylated, the R1 and R2 positions can be distinguished. In order to facilitate the discussion of the SARs we kept the designation R1 and R2 even in the biphenolic compounds, as depicted in Figure 4. Compared to the simple biphenyl 40 (R1, R2 = H) an increase in the size of the substituent in the R2 position resulted in an enhanced affinity of the compounds (46–49). The determined Ki values at CB2 receptors increased from approximately 10 μM for the mono-propyl-substituted compound 46 to 1.16 μM for the pentyl-substituted 48. A further elongation of the alkyl chain to hexyl (49) did not further improve CB2 receptor affinity.

By keeping the residue in the R2-position constant we investigated the influence of the size of the substituent on the other side (R1). The length of the R1 alkyl moiety strongly contributed to the affinity of the magnolol analogs. The rank order of potency at the CB2 receptor for compounds with a hexyl residue at R2 was as follows: R2 = H (49, Ki 1.50 μM)<methyl (53), 0.273 μM<ethyl (57), 0.0830 μM<propyl (61), 0.0294 μM.

Substitution with residues larger than propyl markedly reduced affinity to CB receptors (compare R1 = propyl (61), Ki 0.0294 μM/ R1 = butyl (65), 0.670 μM/R1 = hexyl (45), 1.83 μM). As a next step we kept the favorable propyl residue constant and varied the

Table 2. Activities of Magnolol Derivatives and Standard Compounds at human GPR18 and GPR55

| Compd. | radioligand binding assays vs. [3H]CP55,940 | β-arrestin recruitment assay |
|--------|------------------------------------------|-------------------------------|
|        | CB1 Ki (nM) | CB2 | EC50 or IC50 (μM) | GPR18 | GPR55 |
| 1      | 3.88 | 71.6 | 4.61±0.50 | agonist | antagonist 65% |
| 4      | 1.28 [21] | 1.42 [21] | 5.99±1.88 | agonist | antagonist 188% |
| 5      | 12.6 [34] | 900 [34] | 10.1±1.3 | antagonist | antagonist 94% |
| 6      | 141 [47] | 14900 [47] | 4.97±1.51 | antagonist | antagonist 81% |
| 7      | 5150 [2,19] | 31.2 [2,19] | ~ 10 | antagonist | antagonist 52% |
| 11     | 8340 | 43.3 | >10 | antagonist | antagonist 48% |
| 12     | 2260 | 416 | 30.9±15.8 | antagonist | antagonist 82% |
| 12a    | 267 | 221 | >10 | antagonist | antagonist 41% |
| 60     | 362 | 37.1 | 14.5±2.8 | >10 | |
| 60a    | 17.3 | 31.0 | >10 | antagonist | antagonist 47% |
| 61     | 145 | 29.4 | 10.4±1.1 | >10 | |
| 61a    | 9.57 | 23.8 | >10 | antagonist | antagonist 139% |
| 61b    | 313 | 281 | >10 | antagonist | antagonist 22% |

*a all data result from three independent experiments, performed in duplicates.

**% inhibition of Δ2-THC (10 μM)-induced β-arrestin recruitment by test compounds at a concentration of 10 μM.

**% inhibition of Δ1 (1 μM)-induced β-arrestin recruitment by test compounds at a concentration of 10 μM.

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size of alkyl residues in the \( R^2 \) position from H (46) to octyl (63). Enlargement as well as reduction of the hexyl moiety in the \( R^2 \) position decreased affinity for CB receptors (compare 46, 54, 12 and 58–63). Thus the optimal alkyl combination was obtained by an introduction of the medium-length propyl on the one side and the longer hexyl moiety on the other side of the biphenyl core (61). Compound 61 exhibited \( K_i \) values of 0.145 \( \mu \text{M} \) and 0.0294 \( \mu \text{M} \) at CB1 and CB2 receptors, respectively. The variation of the alkyl residues did not affect the preference of the compounds for CB2 receptors. The highest selectivity (≈25 fold) was obtained when \( R^1 \) was substituted with an ethyl and \( R^2 \) with a butyl residue (55).

The replacement of one hydroxyl by a methoxy group led to a markedly increased CB1 receptor affinity of the magnolol analogs, and at the same time the selectivity towards CB2 receptors was lost, as shown for methoxytetrahydromagnolol (12a). To further investigate this effect we introduced a methoxy group into the two most potent compounds of the present series (60 and 61) leading to the derivatives 60a, 61a and 61b. Like methoxytetrahydromagnolol (12a) the methylated compound 60a lost its selectivity for CB2 receptors; it displayed a 21-fold higher affinity towards the CB1 receptor compared to the parent biphenyl 60, while CB2 receptor affinity was barely affected.

The position of the methoxy group had a big impact on affinity and selectivity, as demonstrated for 61 (Figure 5). The introduction of a methoxy group in the \( \text{para} \)-position to the hexyl residue (61b) drastically reduced the affinity towards CB2 receptors (from a \( K_i \) of 0.0294 \( \mu \text{M} \) to 0.234 \( \mu \text{M} \), Figure 5A), while the decrease in affinity was less pronounced at CB1 receptors (2-fold reduced affinity, Figure 5B). When the methoxy group was introduced in the \( \text{para} \)-position of the short propyl residue a remarkable boost in CB1 receptor affinity could be observed leading to the most potent compound of the synthesized series (61a). Compared to the unmethylated 61, 61a exhibited a 15-fold increase in CB1 receptor affinity (Figure 5A), while CB2 receptor affinity was virtually unaltered (Figure 5B). The impact of the methoxy group on CB receptor affinity was even more pronounced when the two structural isomers 61a and 61b were compared. While 61b (methoxy group in the \( \text{para} \)-position with regard to the hexyl chain) exhibited \( K_i \) values of 0.313 \( \mu \text{M} \) and 0.281 \( \mu \text{M} \) at CB1 and CB2 receptors, respectively, 61a (methoxy group in the \( \text{para} \)-position with respect to the propyl residue) displayed a 33-fold increase in CB1 (\( K_i \) 0.00957 \( \mu \text{M} \)) and 12-fold increase in CB2 receptor affinity (\( K_i \) 0.0238 \( \mu \text{M} \)).

Thus the methylation of the phenolic hydroxyl group abolished the preference of magnolol analogs for CB2 receptors and, depending on which of the two phenolic groups was methylated, the resulting compounds possessed an increased CB1 receptor affinity (12a, 60a, 61a) or decreased affinity at both receptor subtypes (61b) compared to the parent biphenyl compounds.

**Functional Properties**

Receptor ligands may exhibit full agonistic, partial agonistic, antagonistic or inverse agonistic activity. In order to study the intrinsic activity of the new magnolol analogs at the \( G_{i/o} \)-coupled CB1 and CB2 receptor subtypes, their inhibitory effects on forskolin-stimulated adenylate cyclase activity was determined in cAMP accumulation assays at a concentration of 10 \( \mu \text{M} \), and compared to the maximal effect (set at 100%) achieved with the full CB1 and CB2 agonist CP55,940 (4 at 1 \( \mu \text{M} \)). For the most potent compounds full concentration-response curves were recorded and \( EC_{50} \) values were determined. The obtained results are presented in Table 1 and in Table S1. In addition, we investigated the previously published CB2-selective 4′-O-methyl-honokiol (11) for its intrinsic activity in cAMP accumulation assays [35]. While the radioligand binding results obtained in our laboratory for 11 were in accordance with the previously published data, the determined functional properties are divergent [35]. Schuehly et al. had reported inverse agonistic effects of 11 as determined in forskolin-induced cAMP accumulation assays in CHO-K1 cells stably expressing the human CB2 receptor. However, in our hands 11 behaved as an agonist at both CB receptor subtypes.

Since in both laboratories cell lines and the applied assay system were similar, the observed divergence in intrinsic activities may be based on different levels of constitutively active receptors in the used cell lines. For the human CB2 receptor it has been shown that a ligand can behave as an inverse or a partial agonist, depending on the fraction of constitutively active receptors [42,43]. In case of a high fraction of constitutively active receptors a low efficacy ligand will behave as an inverse agonist, while the same compound can act as a partial agonist in a system with lower levels of constitutively active receptors [42,43,44]. This well described phenomenon is referred to as ‘protean agonism’ and may explain the divergent intrinsic activities for 11 observed in both laboratories [44].

The naturally derived lead structure magnolol (9) exhibited partial agonistic activities at both receptor subtypes with somewhat higher efficacy at CB1 receptors than at CB2, but higher potency at CB2 receptors [34]. The compounds of the present series displayed, in general, a comparable profile. Efficacy of the synthesized compounds could be modified by variation of the alkyl chain length. The combination of a propyl residue in the \( R^1 \)-position and a pentyl (60) or a hexyl (61) moiety in the \( R^2 \)-position resulted in full agonistic effects at CB1 and almost full efficacy at CB2 receptors (Figure 6). Based on these two compounds the influence of the alkyl chain length on efficacy may be demonstrated. Residues in the \( R^2 \)-position shorter than pentyl or longer than hexyl, in combination with a propyl residue in the \( R^1 \)-position led to a partial agonistic activity at the CB2 receptor subtype (compare 12 and 58–63), while efficacy at the CB1 receptor was barely affected (except for compound 59). The variation of the propyl side chain in the \( R^1 \)-position, in combination with a hexyl residue in the \( R^2 \)-position also decreased CB2 receptor efficacy, leading to CB2 receptor partial agonists. Again, efficacy at the CB1 receptor was virtually unaltered (compare 49, 53, 57, 61, 65). Full agonists at CB1 and CB2 receptors could be obtained by methylation of the hydroxyl group in the \( \text{para} \)-position to the propyl residue (12a, 60a, 61a; Figure 6). In contrast, methylation of the hydroxyl group in the \( \text{para} \)-position of the hexyl residue (61b) resulted in CB1-antagonistic activity and partial agonistic activity at CB2 receptors, emphasizing the importance of the free phenolic hydroxyl group for high intrinsic activity, i.e. efficient receptor activation by the synthesized compounds (Figure 6).

**Selectivity Towards the Related Orphan Receptors GPR18 and GPR55**

It is well known that several cannabinoid receptor ligands interact with the orphan receptors GPR18 and GPR55 [12,45]. Tetrahydromagnolol (12), for example, was recently identified as a weak GPR55 receptor antagonist [34]. As the physiological role of GPR18 and GPR55 is poorly understood, an interaction of newly synthesized CB ligands with these orphan receptors should be considered as it may lead to unpredictable and undesired off-target effects [46]. Thus, previously as well as newly developed CB receptor ligands should also be investigated for an interaction with these related targets. Furthermore, compounds identified to
interact with one of the orphan GPCRs, might serve as useful starting point for the development of urgently needed potent and selective GPR18 or GPR55 receptor ligands.

Thus, we investigated some commercially available and broadly used CB receptor ligands as well as all of the synthesized magnolol derivatives and analogs for potential interaction with these orphan receptors (for complete results see Supporting Information). The results for the most potent CB receptor ligands of the present series as well as for standard CB receptor ligands are summarized in Table 2. All of the investigated commercially available CB receptor ligands - the nonselective agonists CP55,940 (4) and Δ⁹-THC (1), the CB₁-selective antagonists rimonabant (5) and LY320135 (6) [47], and the CB₂-selective antagonist AM630 (7) - displayed potency at least at one of the two investigated orphan receptors. CP55,940 (4) showed inverse agonistic activity at GPR18 and antagonistic activity at GPR55 in the low micromolar range. As already reported by others we could confirm that Δ⁹-THC (1) acted as an agonist at GPR18 (EC₅₀ 4.61 µM) and a
weak antagonist at GPR55 (IC50 14.2 μM) [48,49]. Rimonabant (5) showed an opposite activity profile compared to Δ9-THC (1) acting as a weak agonist at GPR18 (IC50 10.1 μM) but as an agonist at GPR55 [EC50 2.01 μM] [34]. Contrary to the diarylpyrazole derivative rimonabant (5), the benzofuran derivative and CB1 receptor antagonist LY320135 (6) displayed antagonism at both orphan receptors with IC50 values in low micromolar range (IC50 GPR18:4.97 μM; IC50 GPR55:4.14). The CB2-selective antagonist AM630 (7), an indole derivative, was a weak antagonist at GPR18 and GPR55 displaying IC50 values in the range of 10 μM (Table 2).

None of the compounds of the magnolol-derived series showed agonistic activity at the investigated orphan receptors GPR18 and GPR55 (see Table S2). But some of the synthesized compounds inhibited Δ9-THC (10 μM)-mediated β-arrestin recruitment at GPR18 at a test concentration of 10 μM. In the presence of these compounds the measured β-arrestin recruitment was even lower than the basal level, indicating that these compounds acted as inverse agonists. However, concentration-response curves revealed only moderate antagonistic potency (IC50 >10 μM) of the compounds. The compound with the highest antagonistic potency turned out to be 61. Due to limited solubility of the compound the antagonistic potency could only be estimated by extrapolation of the concentration-response curve (estimated IC50 value: 10.4 μM). Thus 61 still exhibited an approximately 70-fold selectivity for CB1 and 350-fold selectivity for the CB2 receptor subtype versus GPR18 (Table 2).

Most of the newly synthesized compounds failed to interact with GPR55. However antagonistic potency at GPR55 was found to be markedly increased by methylation of one of the hydroxyl groups (compare 12, 12a; 60, 60a and 61, 61a, 61b) (see Table 2). In particular methylation of the phenolic hydroxyl group in the para-position with regard to the hexyl residue led to the potent GPR55 antagonist 61b with an IC50 value of 3.25 μM, representing a potential new starting point for the development of GPR55 receptor antagonists with improved potency and selectivity.

Conclusions

Based on the natural product magnolol, that was recently discovered to activate CB receptors, we designed and synthesized a series of analogs, and tested them in radioligand binding studies and cAMP accumulation assays at CB1 and CB2 receptors. Compared to the lead structures magnolol and tetrahydromagnolol a more than 230-fold increase in CB1 and a greater than 17-fold increase in CB2 receptor affinity could be achieved. Like the lead structure almost all of the newly synthesized compounds possessed agonistic activity at CB receptors, exhibiting higher efficacy at CB1 than at CB2, but higher potency at CB2 as compared to the CB1 receptor subtype. Potency and efficacy could easily be altered by methylation of one of the phenolic hydroxyl groups (compare 12/12a; 60/60a and 61/61a/61b). Depending on the position of the methoxy group full agonists at both receptors (61a), or compounds with antagonistic activity at CB1 and partial agonistic activity at CB2 could be obtained (61b), thereby emphasizing the versatility of the biphenyl scaffold for the development of CB receptor ligands.

All compounds were tested for activity at the related orphan receptors GPR18 and GPR55 to investigate their selectivity, since many commercially available CB ligands were shown to interact with those orphan receptors. We could demonstrate that the frequently used standard CB ligands 1, 4, 5, 6 and 7 also interact with the orphan receptors GPR18 and GPR55.

In contrast, the new magnolol analogs were found to be, in general, highly selective for CB receptors, as they showed no or only moderate inhibition of GPR18 and GPR55, while none of the compounds were able to activate the orphan receptors. Because of their selectivity for CB receptors over the orphan receptors GPR18 and GPR55 some of the presented compounds can be considered as unique in the class of CB receptor ligands. However we could demonstrate that minor modifications such as the methylation of a phenolic hydroxyl group, could increase inhibitory potency at GPR55. Compound 61b was the most potent GPR55 antagonist of the present series with an IC50 value of 3.25 μM, but it was even more potent at the CB receptors (Ki CB1:0.313 μM; Ki CB2:0.281 μM). Further optimization of this class of compounds towards GPR55 antagonistic activity may lead to the development of potent and selective GPR55 antagonists.

We developed CB1 agonists with selectivity for CB2 receptors such as 2-[2-hydroxy-5-propyl-phenyl]-4-pentylphenol (60, 60a, Ki CB1:0.362 μM; Ki CB2:0.0371 μM) and 2-[2-hydroxy-5-propyl-phenyl]-4-hexylphenol (61, 61a, Ki CB1:0.145 μM; Ki CB2:0.0294 μM). Both compounds were full agonists at CB1, and partial agonists at CB2 receptors (efficacy 60:81%, 61:70%). Furthermore, dual CB1/CB2 full agonists with high potency were obtained, including 2-[2-methoxy-5-propyl-phenyl]-4-pentylphenol (60a, Ki CB1:0.0173 μM; Ki CB2:0.0310 μM) and 2-[2-methoxy-5-propyl-phenyl]-4-hexylphenol (61a, Ki CB1:0.00957 μM; Ki CB2:0.0238 μM). The relatively simple structures, which possess no stereocenters, are easily accessible in a four- to five-step synthetic procedure from common starting materials. The central reaction step is the well-elaborated Suzuki-Miyaura cross-coupling reaction, which is suitable for a combinatorial chemistry approach. Due to their favourable properties further investigation in animal studies is warranted.

Materials and Methods

All commercially available reagents were obtained from various producers (Acros, Aldrich, Fluka, Merck, and Sigma) and used without further purification. Compounds 13–21, 30, and 40 were commercially available. Solvents were used without additional purification or drying, unless otherwise noted. The used petroleum ether had boiling point between 40 and 80°C. The reactions were monitored by thin layer chromatography (TLC) using aluminum sheets with silica gel 60 F254 (Merck). Column chromatography was carried out with silica gel 0.060–0.200 mm, pore diameter ca. 6 nm. Purity of compounds was determined by LC-MS by recording mass spectra on an API 2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer (turbo ion spray ion source) coupled with a Waters HPLC system (Agilent 1100) including a Phenomenex Luna 3μ C18 column. Purity of all tested compounds was ≥95% unless otherwise noted. 1H- and 13C-NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer. CDCl3, DMSO-d6, MeOD-d4, or D2O were used as solvents as indicated below. Shifts are given in ppm relative to the remaining protons of the deuterated solvents used as internal standard (6H, 13C). Melting points were determined on the Buchi melting point apparatus B-545 and are uncorrected.

Syntheses

4-O-Methylhonokiol (11) was synthesized (Figure S6) as described before [35]. The syntheses of compounds 22–35 have been previously described and were conducted according to published procedures. For details see Supporting Information. Magnolol derivatives 12 and 41–45 have been previously described in the literature but were prepared according to a new
procedure described herein. Compounds 36–39 as well as magnolol analogs 46–65 and 12a, 60a, 61a and 61b are new compounds.

General Procedure for the Synthesis of Boronic Acid Derivatives 34–39
A solution of n-butyllithium (1.7 M in hexane, 38 mL) was slowly added to a cooled (-80°C) solution of 30 mmol 2-bromo-4-alkylphenol or 30 mmol of 2-bromo-1-methoxy-4-alkylphenol respectively, in dry ether (80 mL). The mixture was then allowed to warm up and stirred at rt for 2 h under an argon atmosphere. It was then cooled again (-80°C) and trimethyl borate (5.38 mL, 50 mmol) was rapidly added. The mixture was stirred at -80°C for 0.5 h and then at rt for 15 h under an argon atmosphere. Then 20 mL of 2 Maq. HCl solution were added slowly into the ice-cold reaction mixture and the mixture was stirred again for 0.5 h, while the milky white emulsion gradually became clear. The ether layer was then separated and the aqueous layer was extracted with diethyl ether (3 times with 100 mL each). The combined organic extracts were washed with water (150 mL) and dried over anhydrous sodium sulfate (2 g). The organic layer was then removed under reduced pressure. The residual solid was recrystallized from hot diethyl ether : toluene, 3:7) to give 7.0 Hz, CH3, 3H). 13C NMR (126 MHz, CDCl3) δ 150.72 (Cα-O), 150.68 (Cα-O), 153.01 (Cα), 153.17 (Cα), 131.02 (Cα), 130.95 (Cα), 129.62 (Cα), 129.57 (Cα), 123.81 (Cα, 2C), 116.42 (Cα, 2C), 37.14 (ar-CH3), 35.05 (ar-CH2), 31.67 (CH2), 31.61 (CH2), 28.95 (CH2), 24.69 (CH2), 22.57 (CH2), 14.04 (CH3), 13.79 (CH3). LC/ESI-MS (negative mode) m/z 325 [M-H]-, (positive mode) m/z 327 [M+H]+, 100% (Figure S9). Yield 35.2%.

5-Hexyl-5’-propylbiphenyl-2,2’-diol (61a). 1H NMR (500 MHz, CDCl3) δ 7.19 (d, J = 8.3, 2.2 Hz, CHα, 1H), 7.16 (d, J = 2.2 Hz, CHα, 1H), 7.11 (d, J = 8.2. 2.2 Hz, CHα, 1H), 7.08 (d, J = 2.2 Hz, CHα, 1H), 6.97 (d, J = 3.5 Hz, CHα, 1H), 6.95 (d, J = 3.4 Hz, CHα, 1H), 6.28 (s, OH, 1H), 3.88 (s, O-CH3, 3H), 3.87 (s, OH, 2H), 2.62 (t, J = 7.5 Hz, CHα, 2H), 2.58 (t, J = 7.5 Hz, ar-CH2, 2H), 1.71–1.75 (m, CH2-CH2, 4H), 1.40–1.29 (m, CH2-CH2-CH2, 6H), 0.97 (t, J = 7.3 Hz, CH3, 3H), 0.90 (t, J = 6.9 Hz, CH3, 3H). 13C NMR (126 MHz, CDCl3) δ 153.36 (Cα-O), 151.65 (Cα-O), 153.45 (Cα), 131.32 (Cα), 132.49 (Cα), 131.02 (Cα), 129.04 (Cα), 128.89 (Cα), 127.12 (Cα), 126.17 (Cα), 117.30 (Cα), 111.47 (Cα), 56.31 (O-CH3), 37.17 (ar-CH3), 35.16 (ar-CH3), 31.74 (CH3), 31.67 (CH2), 29.04 (CH2), 24.71 (CH2), 22.63 (CH2), 14.09 (CH3), 13.81 (CH3). LC/ESI-MS (negative mode) m/z 325 [M-H]-, (positive mode) m/z 327 [M+H]+, 100% (Figure S9). Yield 35.2%.

Retroviral Transfection
CHO K1 cells stably transfected with the human CB1 and CB2 receptor were generated with a retroviral transfection system as previously described [21]. 48 h after transfection, cells were selected by adding 0.8 mg/ml of G418 to the cell culture medium (DMEM/F12 supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin). After one week the G418 concentration was reduced to 0.2 mg/ml.

5-Pentyloxyl-5’-propylbiphenyl-2,2’-diol (60). 1H NMR (500 MHz, CDCl3) δ 7.12 (d, J = 8.2, 2.2 Hz, CHα, 2H), 7.08 (d, J = 2.1 Hz, CHα, 2H), 6.94 (dd, J = 8.2, 1.1 Hz, CHα, 2H), 5.58 (s, OH, 2H), 2.57 (m, ar-CH2, 4H), 1.68–1.58 (m, CH2, 4H), 1.38–1.29 (m, CH2-CH2, 4H), 0.96 (t, J = 7.3 Hz, CH3, 3H), 0.90 (t, J = 7.0 Hz, CH3, 3H). 13C NMR (126 MHz, CDCl3) δ 150.74 (Cα-O), 150.70 (Cα-O), 150.64 (Cα), 135.77 (Cα), 130.97 (Cα), 130.90 (Cα), 129.70 (Cα), 129.65 (Cα), 129.59 (Cα), 116.39 (Cα), 37.15 (ar-CH3), 35.01 (ar-CH2), 31.47 (CH2), 31.32 (CH2), 24.69 (CH2), 22.49 (CH2), 14.00 (CH3), 13.78 (CH3). LC/ESI-MS (negative mode) m/z 297 [M-H]-, 100% (Figure S7). Yield 26.3%.
Membranes of CHO cells expressing the respective human CB receptor subtype were prepared as previously described [21]. The obtained membrane pellets were resuspended and homogenized in the required amount of 50 mM Tris-HCl buffer, pH 7.4, to obtain a protein concentration of 5–7 mg/mL. Aliquots of the membrane preparation (1 mL each) were stored at −80°C until used.

Radioligand Binding Assays at CB1 and CB2 Receptors

Competition binding assays were performed as described elsewhere using the CB agonist radioligand [3H]-cis-3-[2-hydroxy-4-[1,1-dimethylheptyl]phenyl]-trans-4-[3-hydroxy-propyl] cyclohexanol (CP55,940, 4, final concentration 0.1 nM) [21]. As a source for human CB1 and CB2 receptors membrane preparations of Chinese hamster ovary (CHO) cells stably expressing the respective receptor subtype were used (25 μg of protein per vial for CB1 assays, and 1 μg of protein per vial for CB2 receptor assays, respectively). Stock solutions of the test compound were prepared in DMSO. The final DMSO concentration in the assay was 2.5%. Data were obtained from three independent experiments, performed in duplicates. Data were analyzed using Graph Pad Prism Version 4.02 (San Diego, CA, USA).

A solution of 4-alkylphenol (20 mmol) in chloroform (20 mL), sodiumhydrogencarbonate (2 g, 24 mmol) was added. The resulting suspension was cooled to 0°C. While a solution of elemental bromine (1.12 mL, 22 mmol) in chloroform (8 mL) was slowly added, the suspension was vigorously stirred. After completion of the reaction, monitored by TLC the suspension was filtered. The filter with the solid residue was rinsed once with 50 mL of chloroform. The combined organic solutions were evaporated under reduced pressure. The final workup of the product was done either by distillation or by column chromatography (petroleum ether : ethyl acetate, 9: 1).

Supporting Information

Figure S1 Bromination of para-substituted phenols. To a solution of 4-alkylphenol (20 mmol) in chloroform (20 mL), sodiumhydrogencarbonate (2 g, 24 mmol) was added. The resulting suspension was cooled to 0°C. While a solution of elemental bromine (1.12 mL, 22 mmol) in chloroform (8 mL) was slowly added, the suspension was vigorously stirred. After completion of the reaction, monitored by TLC the suspension was filtered. The filter with the solid residue was rinsed once with 50 mL of chloroform. The combined organic solutions were evaporated under reduced pressure. The final workup of the product was done either by distillation or by column chromatography (petroleum ether : ethyl acetate, 9: 1).

Figure S2 Methylation of 2-bromo-4-alkylphenols. A mixture of dichloromethane (50 mL), water (50 mL), phenol (10 mmol), sodium hydroxide (0.6 g, 15 mmol), methyl iodide (1.87 mL, 30 mmol) and benzyl tri-n-butylammonium bromide (0.36 g, 1 mmol) was stirred vigorously at rt for 12 h. The organic layer was then separated and the aq. layer extracted twice with dichloromethane (30 mL portions each). The combined organic extracts were evaporated under reduced pressure. The final workup of the residue was done by column chromatography (petroleum ether : ethyl acetate, 9: 1).

Figure S3 Synthesis of boronic acid derivatives. A solution of n-butyllithium (1.7 M in hexane, 38 mL) was slowly added to a cooled (−80°C) solution of 30 mmol 2-bromo-4-alkylphenol or 30 mmol of 2-bromo-1-methoxy-4-alkylphenol respectively, in dry ether (80 mL). The mixture was then allowed to warm up and stirred at rt for 2 h under an argon atmosphere. It was then cooled again (−80°C) and trimethyl borate (3.58 mL, 50 mmol) was rapidly added. The mixture was allowed to warm up to 0.5 h and then at rt for 15 h under an argon atmosphere. Then 20 mL of 2 M HCl solution were added slowly into the ice-cold reaction mixture and the mixture was stirred again for 0.5 h, while the milky white emulsion gradually became clear. The ethereal layer was then separated and the aqueous layer was extracted with diethyl ether (3 times with 100 mL each). The combined ether extracts were dried (MgSO4) and after filtration the solvent was evaporated under reduced pressure. The residual solid was recrystallized from hot diethyl ether : toluene, 3:7) to give a white solid.

Figure S4 Suzuki cross-coupling. A solution of toluene (25 mL), ethanol (5 mL) and water (5 mL) in a pressure flask was flushed with argon. While keeping a positive pressure of argon 42 mmol of boronic acid, 42 mmol of 2-bromo-4-alkylphenol or 42 mmol of 2-bromo-1-methoxy-4-alkylphenol respectively, 12.3 mmol (1300 mg) of Na2CO3 and 0.108 mmol (125 mg) of tetrakis(triphenylphosphine)palladium(0) were added. The pressure flask was closed and the mixture was stirred for 18 h at 100°C. The aqueous layer was then separated and extracted three
times with ethyl acetate (80 mL portions each). The combined organic extracts were evaporated under reduced pressure. The final workup of the residue was done by column chromatography (petroleum ether : ethyl acetate = 9: 1).

(TIF)

Figure S5 Demethylation. A solution of 14 mmol of methylated magnolol analog in dry dichloromethane (60 mL) under an argon atmosphere was cooled to −80 °C. While the solution was stirred constantly, 15 mmol of BBr3 (15 mL of a 1 M solution in benzene) was added while the solution was at 0 °C and then allowed to warm up to 0 °C. Then 120 mL of water were added while the solution was at 0 °C. The aqueous layer was then separated and extracted three times with dichloromethane (50 mL portions each). The combined organic extracts were evaporated under reduced pressure. The final workup of the residue was done by column chromatography (petroleum ether : ethyl acetate = 9: 1).

(TIF)

Figure S6 Synthesis of 4’-O-methylhonokiol. Me3SO4 (17 μl, 0.18 mmol) was added to a solution of honokiol (40 mg, 0.15 mmol) in an aqueous KOH solution (5 mL, 10%) and stirred for 1 h at 95 °C. After cooling to rt HCl (1 M, 0.5 mL) was added and the mixture was subsequently extracted with chloroform (5 mL portions each). The organic layers were dried over Na2SO4 and the mixture was subsequently extracted with chloroform (5 mL portions each). The combined organic extracts were evaporated under reduced pressure. The residue was subjected to HPLC separation (see below).

(TIF)

Figure S7 LC/ESI-MS spectrum of 60 (mass spectrum in the positive and negative mode), HPLC chromatogram (HPLC-DAD measured from 220–400 nm) of 60, and its purity determined by HPLC-DAD from 220–400 nm (100%).

(TIF)

Figure S8 LC/ESI-MS spectrum of 61 (mass spectrum in the positive and negative mode), HPLC chromatogram (HPLC-DAD measured from 220–400 nm) of 61, and its purity determined by HPLC-DAD from 220–400 nm (100%).

(TIF)

Figure S9 LC/ESI-MS spectrum of 61a (mass spectrum in the positive and negative mode), HPLC chromatogram (HPLC-DAD measured from 220–400 nm) of 61a, and its purity determined by HPLC-DAD from 220–400 nm (100%).

(TIF)

Figure S10 LC/ESI-MS spectrum of 61b (mass spectrum in the positive and negative mode), HPLC chromatogram (HPLC-DAD measured from 220–400 nm) of 61b, and its purity determined by HPLC-DAD from 220–400 nm (100%).

(TIF)

Table S1 Potencies and Efficacies of Magnolol Derivatives and Analogos at human Cannabinoid Receptor Subtypes*. "all data resulted from three independent experiments, performed in duplicates. *efficacy at 10 μM compared to max. effect of the full agonist CP55,940 (1 μM) = 100%. **e% inhibition of radioligand binding at 10 μM. "nd = not determined.

(DOCX)

Table S2 Activities of magnolol analogs and standard cannabinoid receptor ligands at human GPR18 and GPR55*. *all data result from three independent experiments, performed in duplicates. *effect of test compounds (10 μM) on β-arrestin recruitment at human GPR18 is related to the effect of Δ9-THC in a concentration of 10 μM = 100%. "effect of test compounds (10 μM) on β-arrestin recruitment at human GPR55 is related to the effect of LPI in a concentration of 1 μM = 100%. "n.d. = not determined.

(DOCX)

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Author Contributions

Conceived and designed the experiments: AF VR CEM. Performed the experiments: AF VR CEM. Analyzed the data: AF VR CEM. Contributed reagents/materials/analysis tools: AF VR CEM. Wrote the paper: AF VR CEM.

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