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Antagonists for the orphan G protein-coupled receptor GPR55 based on a coumarin scaffold

Viktor Rempel, [#] *Nicole Volz,* [§] *Franziska Gläser,* [§] *Martin Nieger,* [&] *Stefan Bräse,* [§] *and Christa E. Müller*[#,]*

[#]PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical Chemistry I, An der Immenburg 4, D-53121 Bonn, Germany; [§]Institute of Organic Chemistry, University of Karlsruhe, Karlsruhe Institute of Technology (KIT), Fritz-Haber-Weg 6, D-76131 Karlsruhe, Germany; [&]Laboratory of Inorganic Chemistry, Department of Chemistry, P.O. Box 55 (A.I.Virtasen aukio 1), FIN-00014 University of Helsinki, Finland.

Abstract

The orphan G protein-coupled receptor GPR55, which is activated by 1-lysophosphatidylinositol and interacts with cannabinoid (CB) receptor ligands, has been proposed as a new potential drug target for the treatment of diabetes, Parkinson's disease, neuropathic pain, and cancer. We applied β-arrestin assays to identify 3-substituted coumarins as a novel class of antagonists, and performed an extensive structure-activity relationship study for GPR55. Selectivity versus the related receptors CB_1, CB_2 and GPR18 was assessed. Among the 7-unsubstituted coumarins selective, competitive GPR55 antagonists were identified, such as 3-(2-hydroxybenzyl)-5-isopropyl-8-methyl-2*H*-chromen-2-one (**12**, PSB-SB-489, IC_{50} 1.77 μM, pA_2 0.547 μM). Derivatives with long alkyl chains in position 7 were potent, possibly allosteric GPR55 antagonists which showed ancillary CB receptor affinity. 7-(1,1-Dimethyloctyl)-5-hydroxy-3-(2-hydroxybenzyl)-2*H*-chromen-2-one (**69**, PSB-SB-487, IC_{50} 0.113 μM, K_B 0.561 μM) and 7-(1,1-dimethylheptyl)-5-hydroxy-3-(2-hydroxybenzyl)-2*H*-chromen-2-one (**67**, PSB-SB-1203, IC_{50} 0.261 μM) were the most potent GPR55 antagonists of the present series.

Keywords

GPR18, GPR55, orphan GPCR, cannabinoid receptors, coumarin, antagonist, Δ^9-tetrahydrocannabinol

Introduction

The various physiological effects of natural and synthetic cannabinoids cannot be fully explained by their interaction with the two known cannabinoid (CB) receptor subtypes, CB_1 and CB_2.[1-3] An intensive search for additional CB receptor subtypes resulted in the identification of the orphan receptor GPR55 as a promising candidate for a third CB receptor subtype.[4, 5] Like the CB receptors, GPR55 belongs to the rhodopsin-like seven transmembrane G protein-coupled receptor (GPCR) superfamily.[6] Despite its low amino acid identity with CB_1 (13.5%) and CB_2 (14.4%) several cannabinoids were found to interact with GPR55, including the CB_1-selective antagonist/inverse agonist rimonabant (**1**), the non-selective CB_1/CB_2 full agonist CP55,940 (**2**), and the partial CB agonist Δ^9-tetrahydrocannabinol (Δ^9-THC, **3**).[3, 7-9]

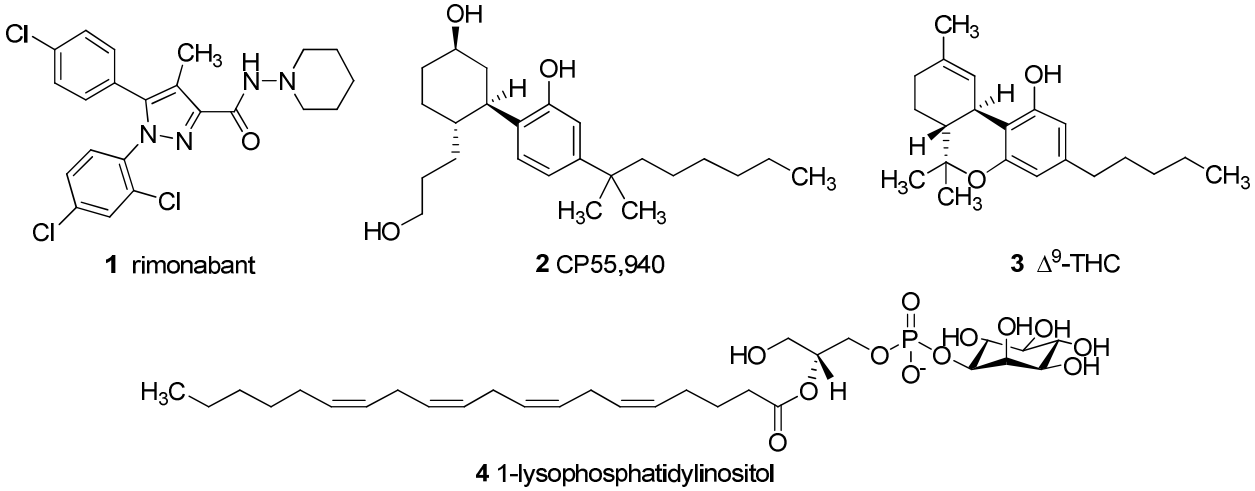


Figure 1. Structures of cannabinoids with GPR55 activity (**1-3**) and the endogenous GPR55 agonist **4**.

Surprisingly, those ligands display opposite effects at the GPR55 as compared to CB receptors: the CB_1 antagonist **1** was found to activate GPR55[9], while the CB agonists **2**[9] and **3**[7] display antagonistic effects at GPR55. Thus, GPR55 has been referred to as an "anti-cannabinoid"

receptor.[10] GPR55 and CB_2 receptors have recently been shown to interact and cross-talk with each other on the level of their signaling pathways, either synergistically or contrarily.[11, 12] In addition, GPR55 and CB_1 receptors were shown to form receptor heteromers thereby modulating each others function.[13]

Nevertheless the classification of GPR55 as a CB receptor is highly controversial. The pharmacology of further cannabinoids, especially that of the endocannabinoids anandamide (arachidonoylethanolamide) and 2-arachidonoylglycerol at GPR55, is not clear.[2, 8, 14] Ryberg et al. reported an activation of GPR55 by anandamide and 2-arachidonoylglycerol, while several other research groups could not detect agonistic effects by these eicosanoids.[3, 9, 15] The only unambiguously confirmed endogenous agonist for GPR55 so far appears to be 1-lysophosphatidylinositol (LPI), a lipid that does not interact with CB receptors.[1, 3, 8] Okuno et al. reported that the biological activity of arachidonic acid-containing LPI species was markedly higher compared to species containing other fatty acids, indicating that 2-arachidonoyl-LPI (**4**) might be the true endogenous agonist for GPR55.[16, 17] Another potential CB receptor subtype might be the orphan GPR18, since some cannabinoids, such as Δ^9-THC and anandamide, were recently shown to interact with that receptor, thereby further emphasizing the complex and not yet fully understood pharmacology of cannabinoids.[18-20]

The GPR55 is a peculiar GPCR since it has not been found to couple to classical G proteins (G_i, G_s, G_q), but only to $G_{12,13}$-proteins thereby activating rhoA (ras homolog gene family member A) and ROCK (Rho-associated protein kinase), which may in turn induce phospholipase C-mediated inositol 1,4,5-triphosphate formation and subsequent calcium release from intracellular stores.[4, 11] In addition, GPR55-mediated activation of the ERK-cascade was confirmed in several studies.[5, 7, 21]

mRNA for the receptor is highly expressed in the brain (highest levels in caudate nucleus, nucleus accumbens, putamen and striatum), on cells and organs of the immune system (lymphocytes, spleen), and in stomach and intestine.[4] High GPR55 expression has also been found on many cancer cells, and GPR55 expression appears to correlate with the proliferation rate and aggressiveness of those cells.[4, 22] Furthermore, inflammatory and neuropathic pain, vasorelaxation and angiogenesis, and bone resorption may be mediated by GPR55.[1, 4] Very recently the LPI/GPR55 system has been found to be positively associated with obesity in humans.[23] Thus, GPR55 is of interest as a novel potential drug target, e.g., for the treatment of diabetes, Parkinson's disease, neuropathic pain, and cancer.[3, 4, 22]

Most of the published pharmacological studies investigating the physiological role of GPR55 were performed with antagonists known to interact with further receptors, such as GPR18 and CB receptors. The lacking selectivity of the utilized compounds considerably limits their suitability as pharmacological tools.[3, 4, 19, 20, 24]

In order to be able to conduct unequivocal in vitro and in vivo studies, and to validate GPR55 as a drug target, appropriate, selective tool compounds are urgently needed to further explore the (patho)physiological roles of GPR55.[4] Recently, the first synthetic GPR55 agonists and antagonists have been identified by the screening of compound libraries, but they have been neither fully characterized nor further optimized.[8, 25] Thus, the goal of the present study was to identify antagonists for GPR55 and to perform extensive studies on their SARs.

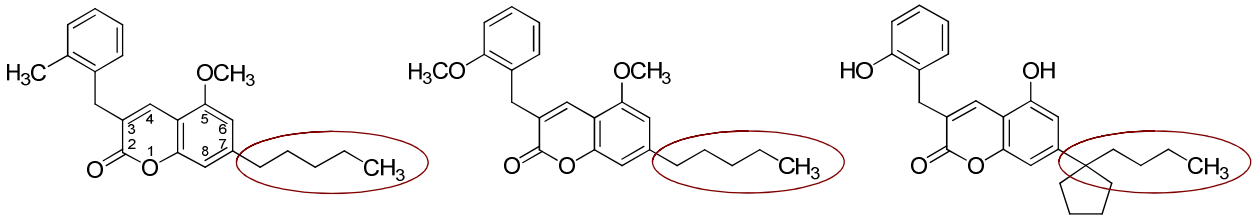
As many of the commercially available cannabinoids did not only activate CB_1 and CB_2 receptors, but were shown to additionally interact with GPR18 and GPR55, our approach was to investigate a series of recently published coumarin-based CB receptor ligands (see Figure 2) as well as inactive analogs of the same series for their potential interaction with both orphan receptors, GPR18 and GPR55. After obtaining several hits for GPR55, but not for GPR18, we

extended the series via optimizing the compounds for interaction with GPR55. This approach led us to identify and develop potent and selective GPR55 antagonists and to carry out an extensive SAR analysis for this pharmacologically attractive receptor.

Results and Discussion

Structural considerations

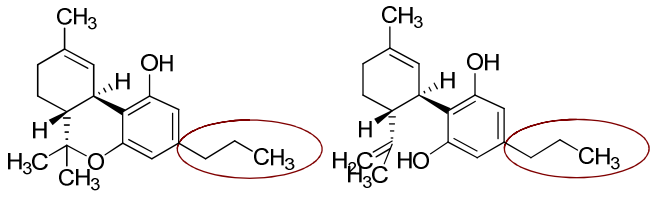
We recently reported on the development of coumarin derivatives as novel ligands for CB receptors.[26, 27] A comparison of the structural features of the 7-alkyl-3-benzylcoumarins **5-7** with recently described GPR55 antagonists isolated from the plant *Cannabis sativa*, Δ^9-tetrahydrocannabivarin (**8**), an analog of **3**, and cannabidivarin (**9**), a cannabidiol analog, is depicted in Figure 2.[7] Compounds **8** and **9** were shown to exhibit antagonistic activity at GPR55: they reduced the maximum effect of LPI-mediated ERK1/2 phosphorylation in human embyonic kidney (HEK) 293 cells stably expressing the human GPR55 by 50% (**8**) and 56% (**9**) at a concentration of 1 μM.[7]



PSB-SB-1201 (**5**)
CB_1 antagonist, K_i 22 nM

PSB-SB-1202 (**6**)
CB_1/CB_2 agonist
K_i CB_1 32 nM, CB_2 49 nM
EC_{50} CB_1 56 nM, CB_2 14 nM

PSB-SB-1204 (**7**)
CB_2 agonist, K_i 68 nM, EC_{50} 48 nM



8 **9**

Figure 2. Structural comparison of recently identified cannabinoid receptor antagonists (**5**) and agonists (**6**, **7**) with the GPR55 antagonists Δ^9-tetrahydrocannabivarin (**8**) and cannabidivarin (**9**).[7, 27]

It becomes evident that the lipophilic side-chain of the GPR55 antagonists **8** and **9** is two methylene units shorter as compared to those in the structurally related, coumarin-based CB receptor ligands **5**-**7**. Since shorter side-chains appeared to be favourable for GPR55 receptor interaction, we decided to include coumarin derivatives with no or with only a short lipophilic substituent in position 7 in our studies, most of which had not shown high affinity for CB receptors in previous investigations.[7, 26] Based on the obtained test results at GPR55 we additionally synthesized a series of new compounds.

Syntheses

Syntheses of 3-methyl- and 3-benzyl-substituted coumarins with modifications in various positions were recently described.[26-30] We established a straightforward procedure to obtain coumarins in a one-pot synthesis from appropriately substituted salicylaldehydes and α,β-unsaturated aldehydes.[26-28] According to this procedure (see Experimental and Supporting Information) further derivatives were obtained with the goal to optimize the compounds for interaction with GPR55. The products were purified by flash chromatography. The structures were confirmed by ^{1}H and ^{13}C NMR spectra, and in some cases by EI-HRMS spectra; purity was confirmed by elemental analysis (for details see Experimental Section and Supporting Information). For one final product, **14**, a crystal structure was obtained (see Supporting Information).

Biological Evaluation

Activities at the human GPR55 were investigated in β-arrestin translocation assays using Chinese hamster ovary (CHO) cells stably expressing the receptor.[3, 9, 21] To monitor β-arrestin recruitment to the activated receptors, the β-galactosidase enzyme fragment complementation technology was applied (β-arrestin PathHunter™ assay, DiscoverX, Fremont, CA, USA). In these cells, the β-arrestin molecule is fused to an inactive deletion mutant of β-galactosidase, which functions as an enzyme acceptor (EA). The investigated receptor is extended by a small low-affinity fragment derived from the sequence deleted in the EA.[31] Activation of the receptor by a ligand will cause recruitment of β-arrestin molecules to the receptor, resulting in a complementation of the two fragments to form a functional enzyme, which is then able to cleave an added detection reagent resulting in light emission.[31] The compounds were initially screened for agonistic and antagonistic activity at the receptor at a concentration of 10 μM. Agonistic effects of test compounds were compared to the effect of the GPR55 agonist LPI at a concentration of 1 μM (set at 100%). IC_{50} values were determined for compounds showing an inhibition of >60% of the LPI signal at 1 μM. Testing of the compounds at the human GPR18 was carried out by the same assay technology. For receptor stimulation, the GPR18 agonist **3** was used at a concentration of 10 μM. The affinities of the 3-benzylcoumarin derivatives at human CB receptors were determined in radioligand binding studies as previously described.[26, 27] As a CB receptor radioligand [^{3}H](-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (**2**) was used. As a source for human CB_1 and CB_2 receptors, membrane preparations of CHO cells stably expressing the respective receptor subtype were utilized. Rat CB_1 receptors were obtained from rat brains as previously described.[26] Initially the

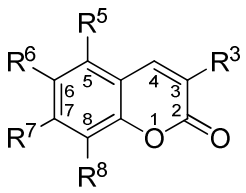
compounds were screened at a concentration of 10 μM. In cases where inhibition of radioligand binding was about 50% or more, full concentration-inhibition curves were determined in order to calculate K_i values. Functional properties at CB receptors were investigated in cAMP assays using CHO cells stably expressing the respective human CB receptor subtype as previously described.[27] Effects of test compounds (1 μM) on forskolin-stimulated cAMP levels were determined relative to the maximal effect observed with the full agonist **2**.

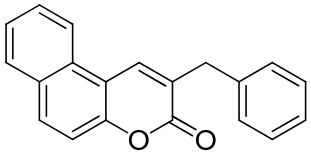
Structure-activity relationships

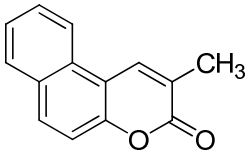
To validate the applied GPR55 test system, we initially investigated some standard ligands. **3** had been reported to be an agonist,[32] an antagonist,[7] or to display no activity at GPR55.[9] In our experiments measuring GPR55-induced β-arrestin recruitment **3** behaved as a moderately potent antagonist at GPR55 (IC_{50} 14.2 μM, see Table 1). We had previously reported that **2** behaved as a relatively potent GPR55 antagonist displaying an IC_{50} value in the low micromolar range (1.61 μM).[33] This is in agreement with results published by other groups.[8, 9] Furthermore, we recently confirmed that **1** acts as a GPR55 agonist[33] (see Table 1) in agreement with literature data.[8, 21]

None of the coumarins investigated in the present study showed agonistic activity at GPR55. However, many coumarin derivatives were found to inhibit LPI-induced GPR55 activation (see Table 1).

Table 1. Potencies of coumarin derivatives at GPR55[a]







24 **25**

Compd	R^3	R^5	R^6	R^7	R^8	β-arrestin assay
						human GPR55 IC_{50} ± SEM (μM)
Standard agonists and antagonists						
4						1.00 ± 0.25 *(agonist, EC_{50})*
1						2.01[33] *(agonist, EC_{50})*
2						1.61[33]
3						14.2 ± 5.4
Coumarin derivatives I: with small 7-substituents						
10	methyl	isopropyl	H	H	methyl	3.45 ± 0.36
11	benzyl	isopropyl	H	H	methyl	5.33 ± 1.10
12	2-hydroxy-benzyl	isopropyl	H	H	methyl	1.77 ± 0.23
13	2-methoxy-benzyl	methyl	H	H	methyl	7.14 ± 2.66
14	2-methoxy-benzyl	methyl	H	H	isopropyl	5.70 ± 1.62
15	benzyl	H	I	H	H	> 10 (46%)[b]
16	methyl	H	I	H	H	> 10 (40%)[b]
17	benzyl	H	H	H	H	> 10 (28%)[b]
18	methyl	H	methoxy	H	H	> 10 (13%)[b]
19	benzyl	H	methoxy	H	H	~ 10 (54%)[b]
20	methyl	H	Cl	H	Br	> 10 (45%)[b]
21	methyl	H	Br	H	methoxy	> 10 (16%)[b]
22	methyl	H	H	H	methoxy	> 10 (7%)[b]
23	benzyl	H	H	H	methoxy	> 10 (5%)[b]

24	see above for structure					2.81 ± 1.16
25	see above for structure					> 10 (28%)[b]
26	benzyl	methoxy	H	H	H	> 10 (43%)[b]
27	benzyl	hydroxy	H	H	H	> 10 (6%)[b]
28	benzyl	methoxy	H	H	Br	> 10 (2%)[b]
29	benzyl	methoxy	H	H	H	9.38 ± 0.58
30	3-methoxy-benzyl	methoxy	H	H	H	~ 10 (54%)[b]
31	3-methoxy-benzyl	methoxy	H	Br	H	3.99 ± 0.75
32	2-methyl-benzyl	hydroxy	H	methyl	H	> 10 (45%)[b]
33	benzyl	methoxy	H	methyl	H	6.74 ± 2.04
34	4-methyl-benzyl	hydroxy	H	methyl	H	> 10 (42%)[b]
35	2-methoxy-benzyl	methyl	methoxy	methyl	methyl	0.981 ± 0.140
36	benzyl	methyl	methoxy	methyl	methyl	12.8 ± 3.2
37	benzyl	methyl	hydroxy	methyl	methyl	9.32 ± 1.05
38	4-fluoro-benzyl	methyl	methoxy	methyl	methyl	13.5 ± 4.3
39	4-fluoro-benzyl	methyl	hydroxy	methyl	methyl	10.3 ± 0.7
40	2-methyl-benzyl	methyl	methoxy	methyl	methyl	7.69 ± 1.71
41	2-methyl-benzyl	methyl	hydroxy	methyl	methyl	5.16 ± 0.73
42	3-methoxy-benzyl	methoxy	H	hydroxymethyl	H	> 10 (13%)[b]
43	3-methoxy-benzyl	methoxy	H	bromomethyl	H	> 10 (18%)[b]
44	benzyl	H	H	methoxy	H	~ 10 (53%)[b]
45	methyl	H	H	methoxy	H	> 10 (25%)[b]
Coumarin derivatives II: 7-pentyl-substitution						
46	4-methoxy-3,5-dimethyl-benzyl	methoxy	H	pentyl	H	> 10 (44%)[b]
47	2-methoxy-benzyl	methoxy	H	pentyl	H	6.35 ± 2.66
48	2-hydroxy-benzyl	hydroxy	H	pentyl	H	> 10 (37%)[b]

49	3-methoxy-benzyl	methoxy	H	pentyl	H	3.23 ± 0.31
50	3-hydroxy-benzyl	hydroxy	H	pentyl	H	10.6 ± 4.9
51	benzyl	methoxy	H	pentyl	H	> 10 (36%)[b]
52	benzyl	hydroxy	H	pentyl	H	> 10 (0%)[b]
53	2-methyl-benzyl	methoxy	H	pentyl	H	5.08 ± 1.05
54	2-methyl-benzyl	hydroxy	H	pentyl	H	> 10 (19%)[b]
55	3-methyl-benzyl	methoxy	H	pentyl	H	> 10 (27%)[b]
56	3-methyl-benzyl	hydroxy	H	pentyl	H	> 10 (19%)[b]
57	2-chloro-benzyl	methoxy	H	pentyl	H	9.00 ± 2.44
58	2-chloro-benzyl	hydroxy	H	pentyl	H	> 10 (0%)[b]
59	3-chloro-benzyl	methoxy	H	pentyl	H	> 10 μM (36%)[b]
60	3-chloro-benzyl	hydroxy	H	pentyl	H	> 10 (30%)[b]
61	4-chloro-benzyl	methoxy	H	pentyl	H	3.29 ± 1.30
62	4-fluoro-benzyl	methoxy	H	pentyl	H	~ 10 (57%)[b]
63	4-bromo-benzyl	methoxy	H	pentyl	H	3.76 ± 1.46
Coumarin derivatives III: long, branched 7-substituent						
64	benzyl	methoxy	H	1,1-dimethylheptyl	H	~ 10 (51%)[b]
65	benzyl	hydroxy	H	1,1-dimethylheptyl	H	0.358 ± 0.089
66	2-methoxy-benzyl	methoxy	H	1,1-dimethylheptyl	H	> 10 (25%)[b]
67	2-hydroxy-benzyl	hydroxy	H	1,1-dimethylheptyl	H	0.261 ± 0.181
68	2-methoxy-benzyl	methoxy	H	1,1-dimethyloctyl	H	> 10 (31%)[b]
69	2-hydroxy-benzyl	hydroxy	H	1,1-dimethyloctyl	H	0.113 ± 0.020
70	2-methoxy-benzyl	methoxy	H	1-butylcylopentyl	H	> 10 (30%)[b]
71	2-hydroxy-benzyl	hydroxy	H	1-butylcylopentyl	H	0.854 ± 0.454
72	2-methoxy-benzyl	methoxy	H	1-butylcylcohexyl	H	> 10 (44%)[b]
73	2-hydroxy-benzyl	hydroxy	H	1-butylcylcohexyl	H	0.961 ± 0.431

[a]All data result from three independent experiments, performed in duplicates
[b]Percent inhibition of LPI (1 μM)-induced β-arrestin recruitment by test compounds (10 μM)

The investigated coumarin derivatives can be divided into three groups according to the residues in position seven of the coumarin scaffold (R^7): the first group consists of coumarins with small residues in that position (H, Br, methyl, hydroxymethyl, bromomethyl, methoxy, **10-45**). The second group contains coumarins with a pentyl residue (**46-63**), and the third group comprises compounds with branched aliphatic residues in position 7 (**64-73**). The nature of the 7-substituent had a big influence on the activity of the coumarin derivatives at GPR55. Even more so, it had been found to be crucial for affinity towards CB receptors.[27] Compounds with no or small moieties in position 7 of the coumarin (coumarin derivatives type I) had been shown to generally exhibit no or only low affinity for CB receptors (see Table 3 and references [8, 25]). In contrast, all compounds of this group, that possessed at the same time an alkyl residue like methyl or isopropyl at the 8-position, were identified as antagonists at GPR55 (Table 1).

Regarding the 3-substituent, different residues from a small methyl (**10**) to a larger, aromatic 2-methoxybenzyl (**13**) were tolerated (e.g. compounds **10**, **13**, **11**, **12**). At the 5-position a substituent appeared to be required since all 5-unsubstituted derivatives were inactive. Methyl, isopropyl, and methoxy residues were well tolerated at C5. Isopropyl substitution at that position conferred GPR55 selectivity versus CB receptors (see below).

The 6-position remained unsubstituted in most cases, but substituents like hydroxy (**41**) and methoxy (**35**) were tolerated when combined with small substituents in other positions. Hydroxy at the 6-position appeared to be slightly, but consistently superior to methoxy substitution (compare **36**/**37**, **38**/**39**, **40**/**41**). An annelated benzene ring in the 5,6-position increased potency (**24**).

In position 7 a substituent was not required, but small residues such as bromo and methyl were accepted and could even somewhat improve potency (compare **30**/**31**, **29**/**33**). Larger, lipophilic

residues in the 7-position were also tolerated and led to an increase in affinity in some cases (see below, coumarin subgroups II and III). Interdependence of substituents in different positions was observed. In case of the benzocoumarins (**24**, **25**) only the 3-benzyl-substituted derivative **24** was active, while the 3-methyl derivative **25** did not display any significant GPR55-inhibitory activity.

The second subgroup of investigated coumarins consisted of derivatives with a pentyl moiety in the 7-position. The presence of a lipophilic residue in that position had been shown to be essential for good affinity at CB receptors, thus all compounds of this subgroup displayed high affinity for both CB receptor subtypes (see Table 3).[27] Coumarins of this subgroup possessing a methoxy group in the 5-position were shown to exhibit higher CB receptor affinity than their hydroxy analogues.[27] Interestingly, the same was true for the potency of the compounds at human GPR55: all compounds with a methoxy moiety in that position showed a somewhat higher antagonistic potency at GPR55 than their hydroxyl-substituted analogs (see Table 3). In the 7-pentyl series (coumarins II) benzyl residues with various substituents were present in the 3-position. Substitution of the benzyl residue was required to achieve good inhibitory activity at GPR55: 3-monosubstitution in the *ortho*-, *meta*-, or *para*-position led to compounds with IC_{50} values in the low micromolar range (e.g. *o*-methoxy (**47**), *o*-methyl (**53**), *m*-methoxy (**49**), *p*-Cl (**61**), *p*-Br (**63**)).

The compounds of the second subgroup displayed a comparable profile as the plant-derived cannabinoid prototype **3** and its synthetic derivative **2**, binding to both CB receptor subtypes and acting as antagonists with moderate potency at GPR55. The difference of the presented series in comparison with **3** or **2** was that the identified GPR55 antagonists showed a complex functional

behavior at CB receptors. For example, GPR55 antagonists that displayed at the same time CB_1/CB_2-agonistic (**47**, **49**), CB_1/CB_2-antagonistic (**50**, **63**) or CB_1-antagonistic/CB_2-agonistic activity (**53**, **61**) were identified (for intrinsic activities of selected compounds see Supporting Information and reference[27]).

Coumarins of series III with branched aliphatic chains in the 7-position, i.e. 1-butylcyclopentyl, 1-butylcyclohexyl, 1,1-dimethylheptyl, or 1,1-dimethyloctyl residues, displayed different structure-activity relationships at GPR55 than their pentyl-substituted analogs (series II). We had previously observed that in this series III 5-hydroxyl-substituted coumarin derivatives were more potent at CB receptors than their methoxy-substituted analogs –in contrast to the 7-pentyl series II (see Table 3).[27] Similar SARs were observed at GPR55: while compounds with a methoxy group in the 5-position showed only weak inhibtory effects on LPI-mediated β-arrestin recruitment, all of the respective hydroxy analogues fully inhibited LPI-induced effects in initial screening assays at 10 μM concentration (for results see Table 1). IC_{50} values of all further investigated coumarin derivatives from series III were in the submicromolar concentration range. The rank order of potency for the 7-substituent was as follows: 1,1-dimethyloctyl (**69**) > 1,1,-dimethylheptyl (**67**) > 1-butylcyclopentyl (**71**) ≥ 1-butylcyclohexyl (**73**). The most potent GPR55 antagonist of the present series was **69** (7-(1,1-dimethyloctyl)-5-hydroxy-3-[(2-hydroxyphenyl)methyl]chromen-2-one) with an IC_{50} value of 0.113 μM. In the 3-position, benzyl as well as 3-hydroxybenzyl was well tolerated.

Mechanism of GPR55 inhibition

The pharmacology of cannabinoids at GPR55 appeared to be complex, and published data are in some cases inconsistent. For instance, in some studies **1** and **3** were found to behave as GPR55

agonists, while in others antagonistic activities were reported for those compounds.[3, 7, 24, 32] A recently published study indicated that some cannabinoids may bind to a binding site distinct from the orthosteric LPI site and thus may represent allosteric rather than orthosteric ligands.[7] Due to the lack of an appropriate radioligand and/or mutagenesis studies these findings have yet to be definitely confirmed.[7] However, an allosteric binding site would explain the inconsistent data concerning the intrinisic activities of some cannabinoids, as allosteric modulation is known to be highly probe-, system- and assay-dependent.[34, 35]

Our data indicate that 7-pentylcoumarins and coumarins with a branched, long alkyl chain in position 7 might also interact with an allosteric binding site of GPR55, while compounds without a lipophilic moiety in that position are most likely to be orthosteric antagonists, indicating that the residue at position R^7 might be crucial for the compounds' binding mode at GPR55. Figure 3 shows examplary curves for one compound of each subgroup. Curves of further compounds (**35**, **47**, **71**) are displayed in Supporting Information. In addition to measuring concentration-dependent inhibition of LPI-induced β-arrestin translocation by antagonists at a single concentration of agonist (1 μM of LPI), we also determined full concentration-response curves for LPI in the presence and absence of the most potent antagonists. As far as the potency and solubility of the coumarin derivatives allowed it, we tested 3-4 different antagonist concentrations. To quantify the antagonistic potency, pA_2 values were calculated by using equiactive agonist concentrations in the absence and presence of antagonist. In case of potentially allosteric modulation the pA_2 value was determined using equiactive agonist concentrations at a level of 30% of the maximal response of the depressed concentration-response curve.[36]

Coumarins with no or only a small substituent in position 7 like **12** and **35** (series I) led to a parallel rightward shift of the agonist curve without affecting its efficacy (Figure 3A and Figure

S2A) thereby indicating competitive antagonism. The corresponding agonist-inhibition curve using 1 μM LPI and a range of antagonist concentrations was also consistent with binding to the orthosteric ligand binding site since the LPI signal was completely inhibited (Fig. 3B and Figure S2B); the curves even went below zero indicating inverse agonism. It appears that GPR55 is already tonically activated to some extent in the employed recombinant CHO cell line, and coumarins **12** and **35** were able to not only block the signal induced by the added GPR55 agonist LPI, but to additionally block the basal activity of the receptor measured in the test system. For the most potent compounds of this subgroup, **35** and **12**, IC_{50} values of 0.981 μM and 1.77 μM were determined. In addition, a K_B-value of 1.87 μM was determined for **35** by applying the Schild regression (see Supporting Information, Figure S3).[36] For **12** the calculated pA_2 value was 0.547 μM. Thus, the potency values obtained for the compounds by different methods were consistent. While **35** showed also some affinity for CB receptors (K_i CB_1 3.66 μM, CB_2 0.338 μM), probably due to its substituent (methyl) in the 7-position, **12** was inactive at CB receptors and thus selective for GPR55 (see Table 3).

The curves determined for **49** belonging to the 7-pentyl-substituted coumarin series II might indicate an allosteric binding mode of **49**, as the antagonist (10 μM) led to a rightward shift of the agonist curve (pA_2 11.1 ± 1.9 μM) and at the same time appeared to reduce the maximal effect of LPI (Figure 3C). A K_B value of 16.5 μM was estimated by linear transformation of the data according to the method of Gaddum (see Supporting Information, Figure S4).[36] The assumption of an allosteric binding mode is further supported by the agonist-inhibition curve displayed in Figure 3D. It can be seen that **49** is not able to fully inhibit the effect of LPI, as the inhibition curve does not reach basal levels. This phenomenon can be observed for partial agonists and

allosteric inhibitors. Since the compounds showed no agonistic effects in screening assays, a partial agonism can be excluded. Thus allosteric modulation might be an explanation for the observed effects. Compound **49** turned out to be the most potent compound of this subgroup II at human GPR55, exhibiting an IC_{50} value of 3.23 μM at the human GPR55. However, it was even more potent at CB receptor, where it showed K_i values in the low nanomolar range (see Table 3 and Figure 3D).

All coumarins with branched aliphatic residues in position 7 (series III) that were identified as GPR55 antagonists completely inhibited the LPI-mediated effect at a high concentration of 10 μM (Table 1). Full concentration-response curves for LPI in the absence and in the presence of various coumarin concentrations showed that increasing concentrations of **69** led to a rightward shift and at the same time a depression of the maximal effect of the agonist-response curve (Figure 3E). A K_B value of 0.561 μM was estimated by application of the method of Gaddum, which was in good accordance to the determined pA_2 value of 0.483 μM (see Supporting Information, Figure S5).[36] Due to a long incubation time of 3.5 h for the antagonist (1 h of preincubation) and 2.5 h for the agonist in the applied assay system, the observed results are probably not due to different dissociation kinetics of both ligands. Rather they may indicate an allosteric mode of GPR55 inhibition. An allosteric mode of action had previously been proposed for other CB ligands, such as **1** and **3**.[7] However to clearly confirm an allosteric binding mode of the investigated compounds, dissociation kinetic studies would have to be performed, which are currently not feasible due to the lack of an appropriate radio- or fluorescent-labelled ligand.[7] Compound **69** was the most potent compound of this subgroup at human GPR55, exhibiting an IC_{50} value of 0.113 μM (Figure 3F).

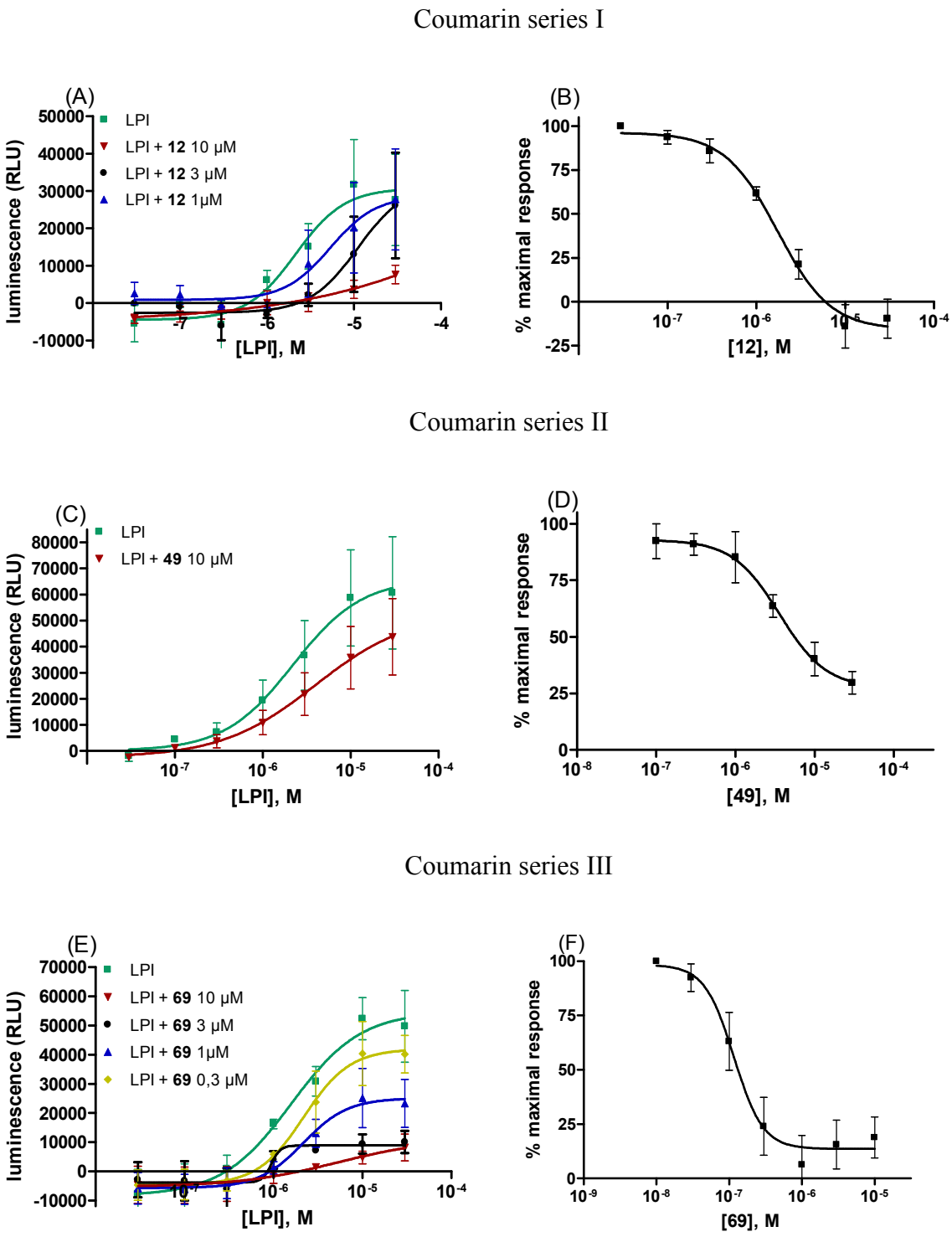


Figure 3. Functional properties of selected coumarin derivatives in β-arrestin recruitment assays performed with CHO cells stably expressing the human GPR55. Compounds with no

lipophilic moiety in position 7 (A, **12** subgoup I) had no impact on E_{max} of LPI and led to a rightwardshift of the agonist curve (see Table 2). A pA_2-value of 0.547 ± 0.119 μM could be determined. Coumarins with a pentyl residue in position 7 (C, **49** subgroup II) exhibited antagonistic properties (pA_2: 11.1 ± 1.9 μM) and a moderate reduction of maximal LPI-resonse, while compounds with branched aliphatic residues (E, **69** subgroup III) displayed considerable concentration-dependent inhibition of LPI's maximal reponse (see Table 2). A pA_2 value of 0.483 ± 0.198 μM could be determined for **69**. Concentration-dependent inhibtion of LPI (1 μM)-response by test compounds revealed IC_{50} values of (B) 1.77 ± 0.23 μM for **12**, (D) 3.23 ± 0.31 μM for **49** and (F) 0.113 ± 0.020 μM for **69**. Data points represent means ± SEMs of three independent experiments, performed in duplicates. For additional curves (compounds **35** (group I), **47** (group II) and **71** (groupIII)) see Supporting Information (Figure S2, S6, and S7).

Table 2. Potencies and maximal effect of LPI in the presence and absence of selected coumarin derivatives at GPR55[a]

Compd	EC_{50} ± SEM of LPI (μM)	$\%E_{max}$
	Coumarin series I	
LPI	2.47 ± 0.89	100
LPI + **12** (1 μM)	10.6 ± 5.3	89 (ns)[b]
LPI + **12** (3 μM)	17.3 ± 6.8	87 (ns)[b]
LPI + **12** (10 μM)	nd[c]	nd[c]
	Coumarin series II	
LPI	2.57 ± 0.89	100
LPI + **49** (10 μM)	4.17 ± 1.47	72 (*)[d]
	Coumarin series III	
LPI	2.25 ± 1.12	100
LPI + **69** (0.3 μM)	3.29 ± 1.28	93 (ns)[b]
LPI + **69** (1 μM)	3.99 ± 1.61	52 (*)[d]
LPI + **69** (3 μM)	0.878 ± 0.386	23 (*)[d]
LPI + **69** (10 μM)	nd[c]	19 (*)[d]

[a]all data result from three independent experiments, performed in duplicates
[b]ns = not significantly different from E_{max}=100%
[c]nd = not determinable
[d]* = significantly different from E_{max}=100% (P-values < 0.05, paired *t*-test)

Selectivity for GPR55

In order to further investigate the selectivity of the compounds they were additionally investigated for an interaction with the orphan receptor GPR18, a putative member of the cannabinoid receptor family.[18, 19] **3** was used as an agonist. In our hands **3** showed an EC_{50} value

of 4.61 μM, which is consonant with literature values (0.96 μM).[19] **1** and **2** behaved as moderately potent GPR18 antagonists (IC_{50} **1**, 10.1 μM; **2**, 5.99 μM).

14, **36**, **39** (subgroup I), as well as **61** (subgroup II), and **65**, **67** and **69** (subgroup III), showed weak antagonistic properties at GPR18 (IC_{50} values ~ 10 μM), while all other compounds failed to display either agonistic or antagonistic activity at GPR18 at a test concentration of 10 μM (see Supporting Information and Table 3). These results indicate that coumarins with short, as well as bulky residues in position 7 may exhibit weak antagonistic activity at the human GPR18. In addition, the results showed that the compounds identified as GPR55 antagonists in the present study are selective versus the putative third CB receptors subtype GPR18. We used exactly the same assay for both receptors, determining agonist-induced β-arrestin translocation by fragment complementation methodology measuring luminescence. This type of assays is very specific, and artefacts are rare.[37] In addition the lack of potency of the coumarin derivatives at GPR18 further proves that the coumarins identified to be GPR55 antagonists exert their effects by direct interaction with the GPR55 receptor protein.

As discussed above, coumarins of series I possessing no substituents in the R^7-position showed generally no or only low CB receptor affinity, while they exhibited remarkable inhibitory effects at GPR55. In particular, coumarins with an additional isopropyl residue at the 5-position displayed high GPR55 selectivity versus CB receptors (**10**, **11**, **12**). Introduction of a methyl residue in position 7 increased affinity for CB receptors, particularly for the CB_2 receptor subtype. 5-Methoxy-coumarins of series II, and 5-hydroxy-coumarins of series III displayed antagonistic effects at GPR55, but showed also high affinity for CB receptors, acting as CB receptor agonists, partial agonists, or antagonists, respectively (see Supporting Information).

Activation of GPR55 and CB receptors is reported to result in contrary physiological effects. For instance, CB_1 and CB_2 receptor agonists have been shown to suppress neuropathic nociception in several models, whereas GPR55 receptor antagonists are considered as potential therapeutics for inflammatory and neuropathic pain.[4, 10, 38, 39] Moreover, CB_1 receptor agonists have been reported to inhibit cancer cell proliferation and migration, while similar effects were reported for GPR55 antagonists.[22, 24, 40] It is tempting to speculate that ligands targeting both receptors, by acting as CB receptor agonists and GPR55 receptor antagonists at the same time, may exhibit additive or even synergistic effects, compared to drugs targeting only one of the respective receptors. Dual receptor ligands featuring both, GPR55-antagonistic and CB-agonistic activity, like **47**, **49** and **65** (GPR55 antagonists, CB_1 and CB_2 agonists) and **67**, **69**, **71** and **73** (GPR55 antagonists, CB_2 agonists) may be interesting pharmacological tools to further explore a therapeutic dual target strategy.

Table 3. Potencies and affinities of selected coumarin derivatives at GPR55, GPR18, CB_1 and CB_2 receptors[a]

Compd	human GPR55	human GPR18	*rat*/human CB_1	human CB_2
	β-arrestin recruitment assay $IC_{50} \pm$ SEM (μM)		*Radioligand binding assays* vs. [^{3}H]CP55,940 $K_i \pm$ SEM (μM)	
Standard CB receptor agonists and antagonists				
1	2.01[33] *Agonist (EC_{50})*	10.1 ± 1.3	0.0126[33]	0.900[33]
2	1.61[33]	5.99 ± 1.88	0.00128[33]	0.00142[33]
3	14.2 ± 5.4	4.61 ± 0.50 *Agonist (EC_{50})*	0.00388 ± 0.00091	0.0716 ± 0.0024
Coumarin derivatives I: with small 7-substituents				
10	3.45 ± 0.36	> 10 (11%)[c]	*> 10 (26%)*[d]	>> 10 (0%)[d]
11	5.33 ± 1.10	> 10 (12%)[c]	*> 10 (24%)*[d]	> 10 (24%)[d]
12	1.77 ± 0.23	> 10 (32%)[c]	> 10 (31%)[d]	> 10 (24%)[d]
13	7.14 ± 2.66	> 10 (0%)[c]	> 10 (16%)[d]	6.83 ± 1.16
14	5.70 ± 1.62	11.3 ± 2.0	> 10 (29%)[d]	0.795 ± 0.254
31	3.99 ± 0.75	> 10 (31%)[c]	> 10 (38%)[d]	2.45[27]
33	6.74 ± 2.04	> 10 (24%)[c]	≥ 10 (45%)[d]	≥ 10 (45%)[d]
35	0.981 ± 0.140	> 10 (27%)[c]	3.66 ± 0.51	0.338 ± 0.146
36	12.8 ± 3.2	≥ 10 (46%)[c]	≥ 10 (42%)[d]	1.77 ± 1.00
37	9.32 ± 1.05	> 10 (16%)[c]	> 10 (21%)[d]	3.42 ± 0.90
38	13.5 ± 4.3	> 10 (32%)[c]	~ 10 (49%)[d]	1.48 ± 0.33
39	10.3 ± 0.7	≥ 10 (47%)[c]	> 10 (34%)[d]	0.444 ± 0.007
40	7.69 ± 1.71	> 10 (33%)[c]	7.86 ± 2.52	0.660 ± 0.171
41	5.16 ± 0.73	> 10 (38%)[c]	≥ 10 (45%)[d]	1.56 ± 0.78
Coumarin derivatives II: 7-pentyl-substitution				
47	6.35 ± 2.66	> 10 (20%)[c]	0.0322[27]	0.0492[27]
49	3.23 ± 0.31	> 10 (33%)[c]	0.0453 [27]	0.143[27]

50	10.6 ± 4.9	> 10 (27%)[c]	16.2[27]	5.15[27]
53	5.08 ± 1.05	> 10 (34%)[c]	0.0222[27]	0.405[27]
54	> 10 (19%)[b]	> 10 (10%)[c]	1.64[27]	3.57[27]
57	9.00 ± 2.44	> 10 (25%)[c]	0.0327[27]	0.185[27]
58	> 10 (0%)[b]	> 10 (0%)[c]	19.1[27]	1.94[27]
61	3.29 ± 1.30	~ 10 (50%)[c]	0.613 ± 0.131	0.578 ± 0.167
63	3.76 ± 1.46	> 10 (30%)[c]	10.2 ± 3.4	0.578 ± 0.136
Coumarin derivatives III: long, branched 7-substituent				
64	~ 10 (51%)[b]	> 10 (31%)[c]	1.43[27]	4.12[27]
65	0.358 ± 0.089	8.10 ± 0.58	2.63[27]	0.465[27]
66	> 10 (25%)[b]	> 10 (26%)[c]	1.02[27]	3.01[27]
67	0.261 ± 0.181	15.9 ± 4.9	0.244[27]	0.210[27]
68	> 10 (31%)[b]	> 10 (33%)[c]	~ 10 (51 %)[d]	~ 10 (47 %)[d]
69	0.113 ± 0.020	12.5 ± 2.9	1.17[27]	0.292[27]
70	> 10 (30%)[b]	> 10 (25%)[c]	0.598[27]	1.14[27]
71	0.854 ± 0.454	≤ 10 (57%)[c]	1.58[27]	0.0676[27]
72	> 10 (44%)[b]	> 10 (33%)[c]	≥ 10 (42 %)[d]	≥ 10 (45 %)[d]
73	0.961 ± 0.431	≤ 10 (59%)[c]	4.89[27]	0.0491[27]

[a]all data result from three independent experiments, performed in duplicates.
[b]% inhibition of LPI (1 μM)-induced β-arrestin recruitment by test compounds in a concentration of 10 μM
[c]% inhibition of **3** (10 μM)-induced β-arrestin recruitment by test compounds in a concentration of 10 μM
[d]% inhibition of radioligand binding at 10 μM

Conclusions

We successfully applied a β-arrestin recruitment assay to identify and characterize novel GPR55 antagonists with a coumarin scaffold and to analyze their SARs. Our results showed that the moiety in position 7 of the coumarin scaffold is crucial for interaction with GPR55, and CB

receptors as well. Compounds with no lipophilic residue in that position possessed in general no or only moderate affinity for CB receptors, and no affinity for the putative third CB receptor subtype GPR18, while they acted as selective, competitive GPR55 antagonists, in particular when combined with a methyl moiety in position 8 of the coumarin scaffold. Thus 8-methylcoumarins represent an appropriate lead structure for the development of more potent and selective GPR55 antagonists in the future. The most potent compound of this subset of compounds, 6-methoxy-3-(2-methoxybenzyl)-5,7,8-trimethyl-2*H*-chromen-2-one (**35**, PSB-SB-258), exhibited an IC_{50} value of 0.981 μM at GPR55, which was in accordance with the determined K_B value of 1.87 μM. 3-(2-Hydroxybenzyl)-5-isopropyl-8-methyl-2*H*-chromen-2-one (**12**, PSB-SB-489, IC_{50} 1.77 μM, pA_2 0.547 μM) and 5-isopropyl-3,8-dimethyl-2*H*-chromen-2-one (**10**, PSB-SB-115, IC_{50} 3.45 μM) are further potent GPR55 antagonist of this subset, possessing selectivity for GPR55.

Introduction of a pentyl residue in position 7 of the coumarin scaffold resulted in a dramatically increased affinity at CB receptors, and a possible shift from orthosteric to allosteric antagonism at GPR55. 5-Methoxy-3-(3-methoxybenzyl)-7-pentyl-2*H*-chromen-2-one (**49**, PSB-SB-435) was the most potent antagonist at GPR55 of this subgroup II (IC_{50} 3.23 μM, pA_2 11.1 μM), possessing at the same time high agonistic potency at both CB receptor subtypes (EC_{50} CB_1: 0.430 μM; CB_2: 0.092 μM, see Supporting Information).

Increase in length and bulk of the alkyl moiety (subgroup III) further increased the antagonistic potency of the compounds at GPR55. 7-(1,1-Dimethyl)alkyl-5-hydroxycoumarins were shown to be very potent antagonists at GPR55 and at the same time agonists at the CB_2 receptor (see Supporting Information). 7-(1,1-Dimethyloctyl)-5-hydroxy-3-(2-hydroxybenzyl)-2*H*-chromen-2-one (**69**, PSB-SB-487, IC_{50} GPR55 0.113 μM, K_B 0.561 μM) and 7-(1,1-dimethylheptyl)-5-

hydroxy-3-(2-hydroxybenzyl)-2*H*-chromen-2-one (**67**, PSB-SB-1203, IC_{50} GPR55 0.261 μM) were found to be the most potent GPR55 antagonists of this subgroup. They may represent interesting tools to study a therapeutic dual target strategy, activating CB_2 receptors and blocking GPR55 at the same time.

Our findings provide a basis for the development of potent GPR55 antagonists with selectivity versus GPR18 and CB receptors, as well as dual GPR55/CB receptor ligands. The coumarins are readily accessible and can easily be modified by a straightforward synthetic procedure. This extensive SAR study of GPR55 ligands along with their detailed characterization may contribute to further elucidating the physiological role of this orphan GPCR, and to explore its potential as a future drug target.

Experimental Section

All commercially available reagents were obtained from various producers and used without further purification. Purity of all tested compounds was ≥95% unless otherwise noted as confirmed by elemental analysis and HPLC-MS measurements. NMR spectra were recorded on a *Bruker* AM 400 (100 MHz), a *Bruker* Avance 300 (300 MHz) or a *Bruker* Avance 400 (400 MHz). Deuterated $CDCl_3$ was used as a solvent unless otherwise noted. The chemical shifts of the remaining protons of the solvent were used as internal standard: ^{1}H, 7.26 ppm; ^{13}C, 77.0 ppm. All chemical shifts (δ) were expressed in ppm. Coupling constants (J) are given in Hertz (Hz). The reactions were monitored by thin layer chromatography (TLC) using aluminum sheets with silica gel 60 F254 (Merck). Elemental analyses were performed in the Institute of Organic Chemistry, University of Karlsruhe with the device *Elementar* vario MICRO. Mass spectra were collected on an Finnigan MAT 95 and IR spectra on a *Bruker* IFS 88.

Syntheses

General procedures for the preparation of coumarin derivatives

Under an atmosphere of argon, 1.00 equiv. of substituted salicylaldehyde, 1.20 equiv. of potassium carbonate, 2.50 equiv. of cinnamaldehyde and 1.20 equiv. of 1,3-dimethylimidazolium dimethylphosphate were suspended in a pressure tube in toluene (7 mL/mmol salicylaldehyde). The reaction mixture was stirred at 200 watt, heated to 110 °C and 7 bar in the microwave for 50 min. It was cooled to room temperature and quenched by addition of water. The product was extracted with ethyl acetate and the combined organic phases were dried over Na_2SO_4, the solvent removed under low pressure and the crude product purified by flash column chromatography.

General procedures for the deprotection of coumarin derivatives

Under an atmosphere of argon, 5.00 equiv. of bortribromide (1 M in dichloromethane) was added drop wise to a solution of 1.00 equiv. of coumarin in dichloromethane (10 mL/mmol coumarin) at – 78 °C. The reaction mixture was stirred for 30 min. at this temperature and was then stirred for another 24 h at room temperature. The reaction was quenched at 0 °C with saturated $NaHCO_3$-solution, extracted with dichloromethane and washed with aqua dest. and brine. The combined organic phases were dried over Na_2SO_4, the solvent removed under low pressure and the product purified by flash column chromatography.

Crystal Structure Determination of 14

The single-crystal X-ray diffraction study was carried out on a Bruker-Nonius Kappa-CCD diffractometer at 123(2) K using MoKα radiation (λ = 0.71073 Å). Direct Methods (SHELXS-97)[41] were used for structure solution and refinement was carried out using SHELXL-97[41] (full-matrix least-squares on F^2). Non hydrogen atoms were refined anisotropically, hydrogen atoms were localized by difference electron density determination and refined using a riding model. A semi-empirical absorption correction was apllied.

14: yellow crystals, $C_{22}H_{24}O_3$, M = 336.41, crystal size 0.50 x 0.35 x 0.25 mm, monoclinic, space group $P2_1/c$ (No. 14), a = 7.5689(6) Å, b = 17.1760(18) Å, c = 14.2218(4) Å, β = 100.855(4)°, V = 1815.(2) Å^3, Z = 4, ρ(calc) = 1.213 Mg m^{-3}, F(000) = 720, μ = 0.081 mm^{-1}, 17360 reflections ($2\theta_{max}$ = 55°), 4136 unique [R_{int} = 0.031], 228 parameters, $R1$ (for 3432 $I > 2\sigma(I)$) = 0.039, *wR2 (all data)* = 0.102, S = 1.02, largest diff. peak and hole 0.278 and -0.233 e Å^{-3}.

Crystallographic data (excluding structure factors) for the structure reported in this work

have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication no. CCDC 930860 (**14**). Copies of the data can be obtained free of charge on application to The Director, CCDC, 12 Union Road, Cambridge CB2 1EZ, UK (Fax: int.code+(1223)336-033; e-mail: deposit@ccdc.cam.ac.uk).

3-(2-Methoxybenzyl)-5,8-dimethyl-2H-chromen-2-one (13). − 73% (Scale: 0.34 mmol) – R_f (cyclohexane/ethyl acetate 10:1) = 0.26. – ^{1}H NMR (400 MHz, [D$_1$]-Chloroform): δ = 2.34 (s, 3H, C8-C*H*$_3$), 2.40 (s, 3H, C5-C*H*$_3$), 3.82 (s, 3H, -OC*H*$_3$), 3.91 (s, 2H, -C*H*$_2$), 6.90 (d, 1H, C3'-*H*, $^3J_{HH}$ = 8.2 Hz), 6.93 (d, 1H, C6-*H*, $^3J_{HH}$ = 7.5 Hz), 6.94 (td, 1H, C5'-*H*, $^3J_{HH}$ = 7.5 Hz), 7.16 (d, 1H C7-*H*, $^3J_{HH}$ = 7.6 Hz), 7.24 – 7.31 (m, 2H, C4'-, C6'-*H*), 7.52 (t, 1H, C4-*H*, $^4J_{HH}$ = 1.2 Hz) ppm. – ^{13}C NMR (100 MHz, [D$_1$]-Chloroform): δ = 15.5 (+, C8-*C*H$_3$), 18.2 (+, C5-*C*H$_3$), 31.3 (–, *C*H$_2$), 55.4 (+, O*C*H$_3$), 110.6 (+, *C*3'), 118.2 (C$_q$, *C*4a), 120.9 (+, *C*5'), 123.6 (C$_q$, *C*8), 125.0 (+, *C*6), 126.4 (C$_q$, *C*4a), 127.4 (C$_q$, *C*1'), 128.3 (+, *C*4'), 131.2 (+, *C*7), 131.6 (+, *C*6'), 132.8 (C$_q$, *C*5), 136.5 (+, *C*4), 152.0 (C$_q$, *C*8a), 157.7 (C$_q$, *C*2'), 162.1 (C$_q$, *C*2) ppm. – IR (Diamant-ATR): 2926 (s, ν OCH$_3$), 1762 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 294 (64) [M$^+$], 252 (26) [C$_{18}$H$_{20}$O$^+$]. − EI-HRMS (C$_{19}$H$_{18}$O$_3$): calc. 294.1256, found 294.1255. – C$_{19}$H$_{18}$O$_3$ (294.1): calc. C 77.53 H 6.16, found C 77.99 H 6.13.

8-(Tert-butyl)-3-(2-methoxybenzyl)-5-methyl-2H-chromen-2-one (14). − 27% (Scale: 1.06 mmol) – R_f (cyclohexane/ethyl acetate 40:1) = 0.22. – ^{1}H NMR (400 MHz, [D$_1$]-Chloroform): δ = 1.43 (s, 3H, C8-C*H*$_3$), 1.48 (s, 9H, C8-C(C*H*$_3$)$_3$), 3.83 (s, 3H, OC*H*$_3$), 3.90 (s, 2H, C*H*$_2$), 6.91 (d, 1H, C3'-H, $^3J_{HH}$ = 8.3 Hz), 6.93 – 6.97 (m, 2H, C5'-, C1-*H*), 7.24 – 7.34 (m, 3H, C4'-, C6'-, C2-*H*), 7.53 (t, 1H, C4-*H*, $^4J_{HH}$ = 1.2 Hz) ppm. − ^{13}C NMR (100 MHz, [D$_1$]-Chloroform): δ = 18.2 (+, C5-*C*H$_3$), 30.1 (+, C(*C*H$_3$)$_3$), 31.2 (–, *C*H$_2$), 34.8 (C$_q$, *C*(CH$_3$)$_3$), 55.4 (+, O*C*H$_3$), 110.6 (+,

*C*3'-H), 118.9 (C_q, *C*4a), 120.9 (+, *C*5'-H), 125.0 (+, *C*6-H), 126.4 (C_q, *C*3), 126.9 (C_q, *C*5), 127.8 (C_q, *C*1'), 127.9 (+, *C*7-H), 128.3 (+, *C*4'-H), 131.4 (+, *C*5'-H), 135.5 (C_q, *C*8), 136.9 (+, *C*4-H), 152.3 (C_q, *C*8a), 157.8 (C_q, *C*2'), 161.4 (C_q, *C*2) ppm. – IR (Diamant-ATR): 2950 (s, ν $C(CH_3)_3$), 2904 (s, ν OCH_3), 1712 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 336 (26) [M^+], 294 (11) [$C_{21}H_{26}O^+$], 259 (39) [$C_{20}H_{19}^+$]. − EI-HRMS ($C_{22}H_{24}O_3$): calc. 336.1725, found 336.1725.

3-Benzyl-5-methoxy-2H-chromen-2-one (26). − 25% (Scale: 1.32 mmol) – R_f (cyclohexane/ethyl acetate 10:1) = 0.21. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 3.88 (s, 3H, OC*H*$_3$), 3.89 (s, 2H, C*H*$_2$), 6.67 (d, 1H, C6-*H*, $^3J_{HH}$ = 8.3 Hz), 6.91 (d, 1H, C8-*H*, $^3J_{HH}$ = 8.3 Hz), 7.24 – 7.34 (m, 5H, Ar-*H*), 7.37 (t, 1H, C7-*H*, $^3J_{HH}$ = 8.3 Hz), 7.78 (s, 1H, C4-*H*) ppm. − ^{13}C NMR (100 MHz, [D_1]-Chloroform): δ = 37.0 (–, *C*H$_2$), 56.0 (+, O*C*H$_3$), 105.1 (+, *C*6-H), 109.0 (+, *C*8-H), 110.2 (C_q, *C*4), 126.8 (+, *C*4'-H), 127.4 (C_q, *C*3), 128.8 (+, *C*3'-, *C*5'-H), 129.3 (+, *C*2'-, *C*6'-H), 131.4 (+, *C*7-H), 134.6 (+, *C*4-H), 138.3 (C_q, *C*1'), 154.4 (C_q, *C*5), 155.9 (C_q, *C*8a), 161.9 (C_q, *C*2) ppm. – IR (Diamant-ATR): 2930 (s, ν OCH_3), 1718 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 266 (100) [M^+], 224 (4) [$C_{16}H_{16}O^+$], 221 (4) [$C_{16}H_{14}O^+$]. − EI-HRMS ($C_{17}H_{14}O_3$): calc. 266.0943, found 266.0945.

3-Benzyl-5-hydroxy-2H-chromen-2-one (27). − 88% (Scale: 5.79 mmol) – R_f (cyclohexane/ethyl acetate 5:1) = 0.17. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 3.90 (s, 2H, C*H*$_2$), 5.24 (s, 1H, O*H*), 6.60 (d, 1H, C6-*H*, $^3J_{HH}$ = 8.2 Hz), 6.90 (d, 1H, C8-*H*, $^3J_{HH}$ = 8.2 Hz), 7.28 – 7.26 (m, 6H, Ar-*H*, C7-*H*), 7.72 (s, 1H, 4-*H*) ppm. − ^{13}C NMR (100 MHz, [D_6]-Acetone): δ = 37.2 (–, *C*H$_2$), 108.0 (+, *C*8-H), 109.9 (C_q, *C*4a), 110.5 (+, *C*6-H), 127.3 (+, *C*4'-H), 127.7 (C_q, *C*3), 129.4 (+, *C*3'-, *C*5'-H), 130.0 (+, *C*2'-, *C*6'-H), 132.3 (+, *C*7-H), 135.1 (+, *C*4-H),

139.6 (C_q, *C*1‘), 155.0 (C_q, *C*8a), 155.4 (C_q, *C*5), 161.8 (C_q, *C*2) ppm. – IR (Diamant-ATR): 3059 (s, ν OH), 1667 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 252 (100) [M^+], 208 (1) [$C_{15}H_{12}O^+$], 131 (2) [$C_9H_7O^+$]. − EI-HRMS ($C_{16}H_{12}O_3$): calc. 252.0786, found 252.0784. – $C_{16}H_{12}O_3$ (252.1): calc. C 76.18 H 4.79, found C 75.72 H 4.85.

6-Methoxy-3-(2-methoxybenzyl)-5,7,8-trimethyl-2H-chromen-2-one (35). − 41% (Scale: 0.27 mmol) − R_f (cyclohexane/ethyl acetate 20:1) = 0.10. − ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 2.28 (s, 3 H, C8-C*H*$_3$), 2.29 (s, 3 H, C7-C*H*$_3$), 2.34 (s, 3 H, C5-C*H*$_3$), 3.65 (s, 3 H, C2’-OC*H*$_3$), 3.82 (s, 3 H, C6-OC*H*$_3$), 2.89 (s, 2 H, C*H*$_2$), 6.90 (d, 1 H, C2’-*H*, $^3J_{HH}$ = 8.2 Hz), 6.94 (td, 1 H, C4’-*H*, $^3J_{HH}$ = 7.4 Hz), 7.23 – 7.30 (m, 2 H, C3’-*H*, C5’-*H*), 7.50 (t, 1H, C4-*H*, $^4J_{HH}$ = 1.2 Hz) ppm. − ^{13}C NMR (100 MHz, [D_1]-Chloroform): δ = 11.1 (+, C7-*C*H$_3$), 11.9 (+, C8-*C*H$_3$), 13.3 (+, C5-*C*H$_3$), 31.2 (–, *C*H$_2$), 55.4 (+, C2’-O*C*H$_3$), 60.6 (+, C4-O*C*H$_3$), 110.6 (+, *C*3’), 116.9 (C_q, *C*4a), 120.9 (+, *C*5’), 123.2 (C_q, *C*8), 124.0 (C_q, *C*5), 126.5 (C_q, *C*3), 126.6 (C_q, *C*1’), 128.2 (+, *C*4’), 131.2 (+, *C*6’), 133.7 (+, *C*7), 136.8 (+, *C*4), 148.4 (C_q, *C*8a), 152.8 (C_q, *C*6), 157.7 (C_q, *C*2’), 162.2 (C_q, *C*2) ppm. − IR (Diamant-ATR): 2917 (s, ν OCH_3), 1706 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 338 (17) [M^+], 296 (44) [$C_{20}H_{22}O_2^+$]. − EI-HRMS ($C_{21}H_{22}O_4$): calc. 338.1518, found 338.1520.

3-Benzyl-6-methoxy-5,7,8-trimethyl-2H-chromen-2-one (36). − 60% (Scale: 1,55 mmol) – R_f (cyclohexane/ethyl acetate 10:1) = 0.24. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 2.30 (s, 6H, C7-,C8-C*H*$_3$), 2.34 (s, 3H, C5-C*H*$_3$), 3.65 (s, 3H, OC*H*$_3$), 3.91 (s, 2H, C*H*$_2$), 7.23 – 7.35 (m, 5H, Ar-*H*), 7.51 (s, 1H, C4-*H*) ppm. − ^{13}C NMR (100 MHz, [D_1]-Chloroform): δ = 11.2 (+, C7-*C*H$_3$), 11.9 (+, C8-*C*H$_3$), 13.4 (+, C5-*C*H$_3$), 36.9 (–, *C*H$_2$), 60.7 (+, O*C*H$_3$), 116.7 (C_q, *C*4a), 123.3

(C_q, *C*8), 124.2 (C_q, *C*5), 126.8 (+, *C*4'-H), 127.4 (C_q, *C*3), 128.8 (+, *C*2'-, *C*6'-H), 129.3 (+, *C*3'-, *C*5'-H), 134.2 (C_q, *C*7), 137.2 (+, *C*4), 138.4 (C_q, *C*1'), 148.5 (C_q, *C*8a), 153.0 (C_q, *C*6), 162.1 (C_q, *C*2) ppm. – IR (Diamant-ATR): 2924 (s, ν OCH_3), 1703 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 308 (1) [M^+]. − EI-HRMS ($C_{20}H_{20}O_3$): calc. 308.1412, found 308.1414.

3-Benzyl-6-hydroxy-5,7,8-trimethyl-2H-chromen-2-one (37). − quant. (Scale: 0.92 mmol) – R_f (cyclohexane/ethyl acetate 5:1) = 0.16. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 2.27 (s, 3H, C8-CH_3), 2.30 (s, 3H, C7-CH_3), 2.38 (s, 3H, C5-CH_3), 3.93 (s, 2H, CH_2), 4.78 (s, 1H, O*H*), 7.25 – 7.38 (m, 5H, Ar-*H*), 7.54 (t, 1H, C4-*H*, $^4J_{HH}$ = 1.1 Hz) ppm. − ^{13}C NMR (100 MHz, [D_1]-Chloroform): δ = 10.8 (+, C7-*C*H_3), 11.9 (+, C8-*C*H_3), 13.0 (+, C5-*C*H_3), 36.9 (–, CH_2), 115.9 (C_q, *C*4a), 116.4 (C_q, *C*5), 122.6 (C_q, *C*8), 126.8 (+, *C*4'-H), 127.2 (C_q, *C*7), 127.5 (C_q, *C*3), 128.8 (+, *C*2'-, *C*6'-H), 129.4 (+, *C*3'-, *C*5'-H), 137.1 (+, *C*4-H), 137.4 (C_q, *C*1'), 146.5 (C_q, *C*8a), 148.2 (C_q, *C*6), 162.2 (C_q, *C*2) ppm. – IR (Diamant-ATR): 3420 (s, ν OH), 1671 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 294 (100) [M^+], 250 (2) [$C_{18}H_{18}O^+$], 147 (5) [$C_{10}H_{11}O^+$]. − EI-HRMS ($C_{19}H_{18}O_3$): calc. 293.1173, found 293.1175.

3-(4-Fluorobenzyl)-6-methoxy-5,7,8-trimethyl-2H-chromen-2-one (38). − 67% (Scale: 1.55 mmol) – R_f (cyclohexane/ethyl acetate 10:1) = 0.19. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 2.32 (s, 3H, C8-CH_3), 2.35 (s, 3H, C7-CH_3), 2.36 (s, 3H, C5-CH_3), 3,68 (s, 3H, OCH_3), 3.89 (s, 2H, CH_2), 7.01 – 7.06 (m, 2H, C3'-, C5'-*H*), 7.27 – 7.31 (m, 2H, C2'-, C6'-*H*), 7.54 (s, 1H, C4-*H*) ppm. − ^{13}C NMR (100 MHz, [D_1]-Chloroform): δ = 11.2 (+, C7-*C*H_3), 11.9 (+, C8-*C*H_3), 13.4 (+, C5-*C*H_3), 36.2 (–, *C*H_2), 60.7 (+, O*C*H_3), 115.5 (+, *C*5'-H), 115.7 (+, *C*3'-H), 116.6 (C_q, *C*4a), 123.4 (C_q, *C*8), 124.2 (C_q, *C*5), 127.2 (C_q, *C*3), 130.7 (+, *C*2'-H), 130.8 (+, *C*6'-H), 134.0 (C_q,

*C*7), 134.4 (C_q, *C*1'), 137.1 (+, *C*4-H), 148.5 (C_q, *C*8a), 153.1 (C_q, *C*6), 161.9 (C_q, *C*2), 161.9 (C_q, *C*4') ppm. – ^{19}F (400MHz, [D_1]-Chloroform): δ = 116.4 ppm. – IR (Diamant-ATR): 2919 (s, ν OCH_3), 1706 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 326 (100) [M^+], 283 (8) [$C_{19}H_{20}FO^+$]. − EI-HRMS ($C_{20}H_{19}FO_3$): calc. 326.1318, found 326.1316.

3-(4-Fluorobenzyl)-6-hydroxy-5,7,8-trimethyl-2H-chromen-2-one (39). − 89% (Scale: 1.01 mmol) – R_f (cyclohexane/ethyl acetate 10:1) = 0.10. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 2.27 (s, 6H, C7-, C8-C*H*$_3$), 2.36 (s, 3H, C5-C*H*$_3$), 3.87 (s, 2H, C*H*$_2$), 4.71 (s, 1H, O*H*), 7.01 (t, 2H, C3'-, C5'-*H*, $^3J_{HH}$ = 8.1 Hz), 7.16 – 7.26 (m, 2H, C2'-, C6'-*H*), 7.52 (s, 1H, C4-*H*) ppm. − ^{13}C NMR (100 MHz, [D_1]-Chloroform): δ = 10.9 (+, C7-*C*H$_3$), 12.0 (+, C7-*C*H$_3$), 13.0 (+, C5-*C*H$_3$), 36.2 (–, *C*H$_2$), 155.5 (+, *C*5'-H), 115.7 (+, *C*3'-H), 115.9 (C_q, *C*4a), 116.3 (C_q, *C*5), 122.7 (C_q, *C*8), 127.3 (C_q, *C*7), 127.3 (C_q, *C*3), 129.1 (C_q, *C*1'), 130.8 (+, *C*6'-H), 130.8 (+, *C*2'-H), 137.0 (+, *C*4-H), 146.6 (C_q, *C*8a), 148.2 (C_q, *C*6), 161.9 (C_q, *C*4'), 162.1 (C_q, *C*2) ppm. – ^{19}F (400MHz, [D_1]-Chloroform): δ = 116.6 ppm – IR (Diamant-ATR): 3456 (s, ν OH), 1673 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 312 (54) [M^+], 269 (5) [$C_{18}H_{18}FO^+$] 147 (5) [$C_{10}H_{11}O^+$]. − EI-HRMS ($C_{19}H_{17}FO_3$): calc. 312.1162, found 312.1160.

6-Methoxy-5,7,8-trimethyl-3-(2-methylbenzyl)-2H-chromen-2-one (40). − 57% (Scale: 1.13 mmol) – R_f (cyclohexane/ethyl acetate 10:1) = 0.29. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 2.13 (s, 3H, C2'-C*H*$_3$), 2.20 (s, 3H, C8-C*H*$_3$), 2.23 (s, 3H, C7-C*H*$_3$), 2.29 (s, 3H, C5-C*H*$_3$), 3.57 (s, 3H, OC*H*$_3$), 3.82 (s, 2H, C*H*$_2$), 7.11 – 7.16 (m, 5H, C4-, C3'-, C4'-, C5'-, C6'-*H*) ppm. − ^{13}C NMR (100 MHz, [D_1]-Chloroform): δ = 11.1 (+, C8-*C*H$_3$), 12.0 (+, C7-*C*H$_3$), 13.4 (+, C5-*C*H$_3$), 19.6 (+, C2'-*C*H$_3$), 34.1 (–, *C*H$_2$), 60.6 (+, O*C*H$_3$), 115.7 (C_q, *C*4a), 123.3 (C_q, *C*8), 124.2 (C_q,

*C*5), 126.5 (+, *C*4'-H), 126.5 (C_q, *C*3), 127.3 (+, *C*5'-H), 130.3 (+, *C*3'-H), 130.7 (+, *C*6'-H), 134.1 (C_q, *C*7), 136.2 (C_q, *C*2'), 136.6 (+,*C*4-H), 136.9 (C_q, *C*1'), 148.3 (C_q, *C*8a), 154.3 (C_q, *C*6), 161.0 (C_q, *C*2) ppm. – IR (Diamant-ATR): 2924 (s, ν OCH_3), 1701 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 322 (100) [M^+], 161 (8) [$C_{11}H_9O_2^+$], 117 (5) [$C_9H_9^+$]. − EI-HRMS ($C_{21}H_{22}O_3$): calc. 322.1569, found 322.1566.

6-Hydroxy-5,7,8-trimethyl-3-(2-methylbenzyl)-2H-chromen-2-one (41). − 73% (Scale: 0.71 mmol) – R_f (cyclohexane/ethyl acetate 5:1) = 0.25. – ^{1}H NMR (400 MHz, [D_6]-Acetone): δ = 2.23 (s, 3H, C2'-C*H*$_3$), 2.29 (s, 3H, C8-C*H*$_3$), 2.31 (s, 3H, C7-C*H*$_3$), 2.32 (s, 3H, C5-C*H*$_3$), 2.85 (s, 2H, C*H*$_3$), 3.85 (s, 1H, O*H*), 7.14 – 7.24 (m, 4H, Ar-*H*), 7.56 (t, 1H, C4-*H*, $^4J_{HH}$ = 1.2 Hz) ppm. ^{13}C NMR (100 MHz, [D_6]-Acetone): δ = 11.1 (+, C7-*C*H$_3$), 11.7 (+, C8-*C*H$_3$), 13.3 (+, C5-*C*H$_3$), 19.6 (+, C2'-*C*H$_3$), 34.5 (–, *C*H$_2$), 117.0 (C_q, *C*4a), 118.2 (C_q, *C*5), 122.5 (C_q, *C*8), 126.9 (+, *C*5'-H), 127.1 (C_q, *C*7), 127.3 (C_q, *C*3), 127.6 (+, *C*4'-H), 129.4 (C_q, *C*2'), 130.58 (+, *C*3'-H), 131.1 (+, *C*6'-H), 137.4 (C_q, *C*1'), 137.6 (+, *C*4-H), 147.0 (C_q, *C*8a), 149.8 (C_q, *C*6), 161.8 (C_q, *C*2) ppm. – IR (Diamant-ATR): 3343 (s, ν OH), 1677 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 308 (100) [M^+], 91 (9) [$C_7H_7^+$]. − EI-HRMS ($C_{20}H_{20}O_3$): calc. 308.1412, found 308.1411.

5-Methoxy-3-(4-methoxy-3,5-dimethylbenzyl)-7-pentyl-2H-chromen-2-one (46). – 42% (Scale: 0.32 mmol) – R_f (cyclohexane/ethyl acetate 5:1) = 0.46. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 0.89 (t, 3H, CH_2CH_3, $^3J_{HH}$ = 6.8 Hz), 1.28 – 1.37 (m, 4H, 2 × C*H*$_2$, C*H*$_2$C*H*$_2$CH$_3$), 1.60 – 1.68 (m, 2H, C*H*$_2$CH$_2$CH$_2$CH$_3$), 2.26 (s, 6H, C3'-, C5'-C*H*$_3$), 2.63 (t, 2H, C7-C*H*$_2$, $^3J_{HH}$ = 7.8 Hz), 3.70 (s, 3H, C4'-OC*H*$_3$), 3.75 (s, 2H, C3-C*H*$_2$), 3.89 (s, 3H, C5-OC*H*$_3$), 6.50 (d, 1H, C6-*H*, $^4J_{HH}$ = 1.3 Hz), 6.72 (d, 1H, C8-*H*, $^4J_{HH}$ = 1.3 Hz), 6.93 (s, 2H, C2'-, C6'-*H*),

7.74 (d, 1H, C4-*H*, $^4J_{HH}$ = 0.9 Hz) ppm. − ^{13}C NMR (100 MHz, [D$_1$]-Chloroform): δ = 14.1 (+, CH_2CH_3), 16.2 (+, C3'-, C5'-CH_3), 22.6 (–, CH_2CH_3), 30.8 (–, $CH_2CH_2CH_3$), 31.5 (–, $CH_2CH_2CH_2CH_3$), 36.3 (–, C7-CH_2), 36.7 (–, C3-CH_2), 55.9 (+, C4'-OCH_3), 59.8 (+, C5-OCH_3), 105.6 (+, *C*8-H), 108.2 (C$_q$, *C*4a), 108.6 (+, *C*6-H), 126.2 (C$_q$, *C*3), 129.5 (+,*C*2'-, *C*6'-H), 130.9 (C$_q$, *C*3'-, *C*5'-CH_3), 133.8 (C$_q$, *C*1'), 134.7 (+, *C*4-H), 147.7 (C$_q$, *C*7), 154.4 (C$_q$, *C*8a), 155.6 (C$_q$, *C*5), 155.7 (C$_q$, *C*3), 162.2 (C$_q$, *C*2) ppm. – IR (KBr): 2989 (s, ν OCH_3), 2926 (s, ν OCH_3), 1726 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 395 (27) [$(M+H)^+$], 394 (100) [M^+], 379 (18) [$C_{24}H_{27}O_4^+$]. − EI-HRMS ($C_{25}H_{30}O_4$): calc. 394.2144, found 394.2146. $C_{25}H_{30}O_4$ (394.2): calc. C 76.11, H 7,66,found C 75.65, H 7.61.

3-(4-Chlorobenzyl)-5-methoxy-7-pentyl-2H-chromen-2-one (61). – 45% (Scale: 0.31 mmol) – R$_f$ (cyclohexane/ethyl acetate 20:1) = 0.27. – ^{1}H NMR (400 MHz, [D$_1$]-Chloroform): δ = 0.89 (t, 3H, CH_3, $^3J_{HH}$ = 6.8 Hz), 1.28 – 1.35 (m, 4H, CH_2CH_2CH$_3$), 1.58 – 1.66 (m, 2H, CH_2CH$_2$CH$_2$CH$_3$), 2.63 (t, 2H, C7-CH_2, $^3J_{HH}$ = 7.8 Hz), 3.82 (s, 2H, C3-CH_2), 3.88 (s, 3H, OCH_3), 6.50 (d, 1H, C6-*H*, $^4J_{HH}$ = 1.3 Hz), 6.72 (d, 1H, C8-*H*, $^4J_{HH}$ = 1.3 Hz), 7.21 – 7.24 (m, 2H, C3'-, C5'-*H*), 7.25 – 7.28 (m, 2H, C2'-, C6'-*H*), 7.73 (d, 1H, C4-*H*, $^4J_{HH}$ = 1.3 Hz) ppm. − ^{13}C NMR (100 MHz, [D$_1$]-Chloroform): δ = 14.1 (+, *C*H$_3$), 22.6 (–, *C*H$_2$CH$_3$), 30.8 (–, *C*H$_2$CH$_2$CH$_3$), 31.5 (–, *C*H$_2$CH$_2$CH$_2$CH$_3$), 36.3 (–, C7-*C*H$_2$), 36.7 (–, C3-*C*H$_2$), 56.0 (+, O*C*H$_3$), 105.7 (+, *C*8-H), 108.0 (C$_q$, *C*4a), 108.6 (+, *C*6-H), 125.5 (C$_q$, *C*3), 128.8 (+, *C*3'-, *C*5'-H), 130.6 (+, *C*2'-, *C*6'-H), 132.5 (C$_q$, *C*4'), 134.9 (+, *C*4-H), 137.0 (C$_q$, *C*7), 148.0 (C$_q$, *C*1'), 154.4 (C$_q$, *C*8a), 155.6 (C$_q$, *C*5), 162.0 (C$_q$, *C*2) ppm. – IR (KBr): 2925 (s, ν OCH_3), 1614 (s, ν C=O) cm^{-1}. – MS (EI): m/z (%) = 372/370 (4/17) [M^+], 316/314 (3/8) [$C_{18}H_{15}^+$]. − EI-HRMS ($C_{22}H_{23}ClO_3$): calc. 370.1336, found 370.1339.

3-(4-Fluorobenzyl)-5-methoxy-7-pentyl-2H-chromen-2-on (62). – 27% (Scale: 0.27 mmol) – R_f (cyclohexane/ ethyl acetate 20:1) = 0.29. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 0.82 (t, 3H, C*H*$_3$, $^3J_{HH}$ = 6.8 Hz), 1.18 – 1.30 (m, 4H, C*H*$_2$C*H*$_2$CH$_3$), 1.51 – 1.58 (m, 2H, C*H*$_2$CH$_2$CH$_2$CH$_3$), 2.56 (t, 2H, C7-C*H*$_2$, $^3J_{HH}$ = 7.9 Hz), 3.75 (s, 2H, C3–C*H*$_2$), 3.80 (s, 3H, OC*H*$_3$), 6.43 (d, 1H, C6-*H*, $^4J_{HH}$ = 1.2 Hz), 6.65 (d, 1H, C8-*H,* $^4J_{HH}$ = 1.2 Hz), 6.92 (t, 2H C2‘-, C6‘-*H*, $^3J_{HH}$ = 8.7 Hz,), 7.24 – 7.29 (m, 2H, C3‘-, C5‘-*H*), 7.64 (q, 1H, C4-*H*, $^4J_{HH}$ = 1 Hz) ppm. – ^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.1 (+, *C*H$_3$), 22.6 (–, *C*H$_2$CH$_3$), 30.8 (–,*C*H$_2$CH$_2$CH$_3$), 31.5 (–,*C*H$_2$CH$_2$CH$_2$CH$_3$), 36.2 (–, C7-*C*H$_2$), 36.7 (–, C3-*C*H$_2$), 56.0 (+, O*C*H$_3$), 105.7 (+, *C*8-H), 108.0 (Cq., C4a), 108.6 (+, C6-H), 115.4 (+, C2‘-, C6‘-H), 115.6 (+, *C*3‘-, C5‘-H), 125.9 (C$_{q.}$, C3), 134.2 (C$_{q.}$, *C*1’), 134.8 (+, *C*4-H), 148.0 (C$_{q.}$, C7), 154.4 (C$_{q.}$, *C*8a), 155.6 (C$_{q.}$, *C*5), 162.0 (C$_{q.}$, C4´), 163.1 (C$_{q.}$, *C*2) ppm. – ^{19}F NMR (400 MHz, $CDCl_3$): δ = 116.6 ppm. – IR (KBr): 2928 (s, ν OCH$_3$), 1700 (s, νC=O) cm^{-1}. – MS (EI, 70 eV): m/z (%) = 355 (22) [(M+H)$^+$], 354 (100) [M$^+$], 298 (42) [C$_{18}$H$_{15}^+$], 109 (9) [C$_7$H$_6$F$^+$], 105 (80) [C$_7$H$_5$O$^+$], 77 (17) [C$_6$H$_5^+$], 55 (3). – EI-HRMS ($C_{22}H_{23}FO_3$): calc. 354.1633, found 354.1631. – $C_{22}H_{23}FO_3$ (354.2): calc C 74.56, H 6.54, found C 74.18, H 6.56.

3-(4-Bromobenzyl)-5-methoxy-7-pentyl-2H-chromen-2-one (63). – 11% (Scale: 0.29 mmol) R_f (cyclohexane/ethyl acetate 5:1) = 0.43. – ^{1}H NMR (400 MHz, [D_1]-Chloroform): δ = 0.89 (t, 3H, C*H*$_3$, $^3J_{HH}$ = 6.8 Hz), 1.29 – 1.36 (m, 4H, C*H*$_2$C*H*$_2$CH$_3$), 1.58 – 1.66 (m, 2 H, C*H*$_2$CH$_2$CH$_2$CH$_3$), 2.63 (t, 2H, C7-*C*H$_2$, $^3J_{HH}$ = 7.9 Hz), 3.81 (s, 2H, C3-C*H*$_2$), 3.88 (s, 3H, OC*H*$_3$), 6.50 (d, 1H, C6-*H*, $^4J_{HH}$ = 1.3 Hz), 6.73 (d, 1H, C8-*H*, $^4J_{HH}$ = 1.3 Hz), 7.16 – 7.20 (m, 2H, C2‘-, C6‘-*H*), 7.41 – 7.45 (m, 2H, C3‘-, C5‘-*H*), 7.64 (d, 1H, C4-*H*, $^4J_{HH}$ = 0.8 Hz) ppm. –

^{13}C NMR (100 MHz, $CDCl_3$): δ = 14.1 (+, *C*H_3), 22.6 (–, *C*H_2CH_3), 30.8 (–, *C*$H_2CH_2CH_3$), 31.5 (–, *C*$H_2CH_2CH_2CH_3$), 36.4 (–, C7-*C*H_2), 36.7 (–, C3-*C*H_2), 56.0 (+, O*C*H_3), 105.7 (+, *C*8), 108.0 ($C_{q.}$, *C*4a), 108.6 (+, *C*6), 120.6 ($C_{q.}$, *C4'*), 125.4 ($C_{q.}$, *C*3), 131.0 (+, *C*2'-, *C*6'-H), 131.8 (+, *C*3'-, *C*5'-H), 134.9 (+, *C*4-H), 137.6 ($C_{q.}$, *C*7), 148.1 ($C_{q.}$, *C*1'), 154.4 ($C_{q.}$, *C*8a), 155.6 ($C_{q.}$, *C*5), 162.0 ($C_{q.}$, *C*3) ppm. – IR (KBr): 2919 (s, ν OCH_3), 1699 (s, ɣC=O) cm^{-1}. – MS (EI, 70 eV): m/z (%) = 416/414 (99/100) [M^+], 415 (26) [$(M+H)^+$], 360/358 (43/44) [$C_{18}H_{15}^+$], 335 (29) [$C_{22}H_{23}O_3^+$]. – EI-HRMS ($C_{22}H_{23}BrO_3$): calc. 414.0830, found 414.0829. – $C_{22}H_{23}BrO_3$ (414.1): calc. 63.62, H 5.58, found. C 63.31, H 5.80.

Retroviral transfection

CHO K1 cells stably transfected with the human CB_1 and CB_2 receptor were generated with a retroviral transfection system as previously described.[27] 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.

Cell culture

GP+envAM12 packaging cells were cultured at 37°C, 5% CO_2 in HXM medium which consists of DMEM, 10% FCS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 1% ultraglutamine, 0.2 mg/ml hygromycin B, 15 µg/ml hypoxanthine, 250 µg/ml xanthine and 25 µg/ml mycophenolic acid. CHO K1 cells were maintained in DMEM/F12 medium with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin under the same conditions. CHO cells stably transfected with

the human CB_1 and CB_2 receptors were maintained at 37° C and 5% CO_2 in the same medium, however in the presence of 0.2 mg/ml G418.

β-Arrestin recruitment assay

Recruitment of β-arrestin molecules to the respective receptor was detected by using β-galactosidase enzyme fragment complementation technology (β-arrestin PathHunter™ assay, DiscoverX, Fremont, CA, USA). CHO cells stably expressing the respective receptor were seeded in a volume of 90 µL into a 96-well plate and were incubated at a density of 20,000 cells/well in assay medium (Opti-MEM, 2 % FCS, 100 U/mL penicillin, 100 µg/mL Streptomycin, 800 µg/mL geneticin und 300 µg/mL hygromycin) for 24 h at 37°C. After the given incubation, test compounds were diluted in PBS buffer containing 10 % DMSO and 0.1 % BSA and added to the cells in a volume of 10 µL, followed by an incubation for 90 min at 37°C. For determination of baseline luminescence PBS buffer (containing 10 % DMSO, 0.1 % BSA) in the absence of test compound was used. During the incubation period, the detection reagent was prepaired. For determination of β-arrestin recruitment to GPR18 the provided detection reagent was used, according to the suppliers protocol. The detection reagent for GPR55 was varied and obtained by mixing the chemiluminescent substrate Galacton-Star® (2 mM), with the luminescence enhancer Emerald-II™ and a lysis buffer (10 mM TRIS, 1 mM EDTA, 100 mM NaCl, 5 mM $MgCl_2$, 1 % Triton-X; pH 8) in a ratio of 1:5:19. After the addition of 50 µL/well of detection reagent to the cells, the plate was incubated for further 60 min at room temperature. Finally luminescence was determined in a luminometer (TopCount NXT, Packard / Perkin-Elmer).

For the determination of antagonistic properties of tested compounds the assay was performed as described for agonists, except the test compounds are added to the cells in a volume of 5 µL/well 60 min prior to addition of the agonist (lysophosphatidylinositol = LPI, 5 µL/well). Data were obtained from three independent experiments, performed in duplicates. Data were analyzed using Graph Pad Prism Version 4.02 (San Diego, CA, USA).

Membrane preparations for CB receptor assays

Membranes of CHO cells expressing the respective human CB receptor subtype were prepared as previously described.[27] The obtained membrane pellets were resuspended and homogenized in the required amount of 50 mM Tris-HCl puffer, 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 CB_1 and CB_2 receptors

Competition binding assays were performed as described elsewhere using the CB agonist radioligand [^{3}H](-)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl) cyclohexanol (**2**), (final concentration 0.1 nM).[27] As a source for human CB_1 and CB_2 receptors membrane preparations of Chinese hamster ovary (CHO) cells stably expressing the respective receptor subtype were used (25 µg of protein per vial for CB_1 assays, and 1 µg of protein per vial for CB_2 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). For the calculation of K_i values the Cheng-Prusoff equation and a K_D value of 2.4 nM ([^{3}H]CP55,940 at human CB_1) and 0.7 nM ([^{3}H]CP55,940 at human CB_2) were used.

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Supporting Information Available: Synthetic procedure and analytical data for all compounds; X-ray structure of compound **14** (CCDC-930860); intrinsic activities of coumarins measured in β-arrestin recruitment assays at the GPR18; intrinsic activities of selected coumarins in cAMP accumulation assays at human CB_1 and CB_2 receptors. Antagonistic activities of **35**, **47** and **71** in β-arrestin recruitment assays at GPR55. This material is available free of charge via the internet at http://pubs.acs.org.

Corresponding Author: *Christa E. Müller, PharmaCenter Bonn, Pharmaceutical Institute, Pharmaceutical Chemistry I, An der Immenburg 4, D-53121 Bonn, Germany. Phone: +49-228-73-2301. Fax: +49-228-73-2567. E-mail: christa.mueller@uni-bonn.de.

Abbreviations

CB, cannabinoid; CHO cells, Chinese hamster ovary cells; EA, enzyme acceptor; ERK, extracellular-signal-regulated kinase; GPR18, G protein-coupled receptor 18; GPR55, G protein-coupled receptor 55; HEK cells, human embryonic kidney cells; LPI, lysophosphatidylinositol; nd, not determined; PSB, Pharmaceutical Sciences Bonn; rhoA, ras homolog gene family member A; ROCK, Rho-associated protein kinase; SARs, structure-activity relationships; THC, tetrahydrocannabinol

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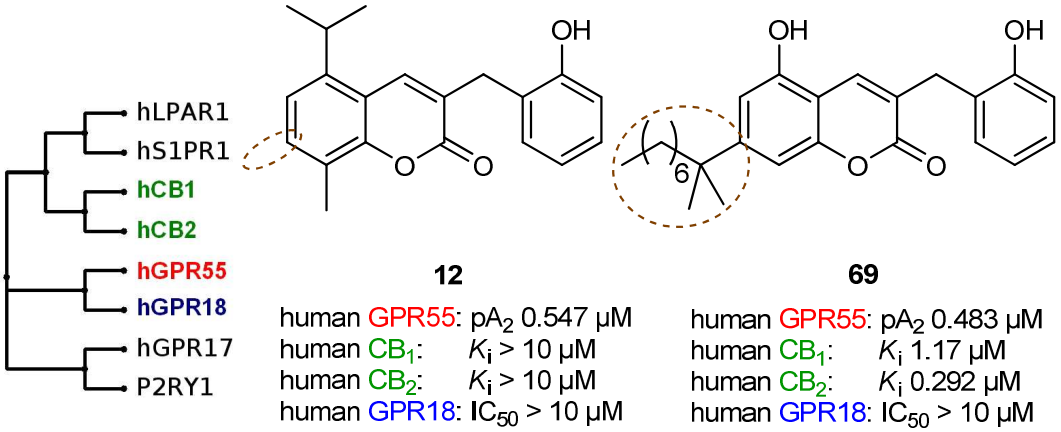

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human GPR55: pA_2 0.547 μM
human CB_1: K_i > 10 μM
human CB_2: K_i > 10 μM
human GPR18: IC_{50} > 10 μM

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human GPR55: pA_2 0.483 μM
human CB_1: K_i 1.17 μM
human CB_2: K_i 0.292 μM
human GPR18: IC_{50} > 10 μM