

Bicyclic imidazole-4-one derivatives: a new class of antagonists for the orphan G protein-coupled receptors GPR18 and GPR55†

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GPR18 and GPR55 are orphan G protein-coupled receptors (GPCRs) that interact with certain cannabinoid (CB) receptor ligands. In the present study bicyclic imidazole-4-one derivatives were discovered as new scaffolds for the development of antagonists for GPR18 and GPR55. Interaction with CB₁ and CB₂ receptors was also studied to assess selectivity. The presented extensive structure–activity relationship study of 49 derivatives investigated at all four GPCRs revealed structural requirements for the development of potent and selective GPR18 and GPR55 antagonists. (*Z*)-(2,3-Difluorobenzylidene)-6,7-dihydro-2*H*-imidazo[2,1*b*][1,3]thiazin-3(5*H*)-one (**18**) was identified as a selective GPR55 antagonist (IC₅₀ 3.15 μM). The most potent GPR18 antagonist was (*Z*)-2-(3-(4-chlorobenzoyloxy)benzylidene)-6,7-dihydro-2*H*-imidazo[2,1*b*][1,3]-thiazin-3(5*H*)-one (**32**, IC₅₀ 0.279 μM, >36-fold selective vs. CB₁ and GPR55, 14-fold selective vs. CB₂) representing the first selective GPR18 antagonist. The new compounds may serve as lead structures and as tools to explore the (patho-)physiological roles of these orphan GPCRs and their potential as drug targets.

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Introduction

Two distinct cannabinoid (CB) receptor subtypes are known to date, designated CB₁ and CB₂, which belong to the G protein-coupled receptor (GPCR) superfamily of 7-transmembrane receptors.¹ Together with their endogenous ligands anandamide (**1**) and 2-arachidonoylglycerol (**2**), and the enzymes responsible for their production and degradation, the cannabinoid receptors are part of the endocannabinoid system, which is involved in a variety of physiological and pathophysiological processes. These include the regulation of appetite and energy homeostasis, cognitive and mental functions, and effects on pain transmission and inflammation.^{2–5} Due to the immense potential of cannabinoids and CB receptor antagonists as

drugs, a variety of synthetic compounds has been developed and pharmacologically characterized.⁶ However, some of the pharmacological studies revealed that not all observed effects caused by cannabinoids could be fully explained by interaction of the compounds with the known CB receptors since some effects were also observed in CB receptor knockout mice. These results indicated that certain cannabinoids are able to interact with further targets.^{3,7}

Recently the orphan receptor GPR18 has been reported to be activated by Δ⁹-THC (**3**) (Fig. 1) as well as by the endogenous CB receptor agonist anandamide (**1**).^{8–11} Cannabidiol (**4**) was found to be a moderately potent partial agonist at GPR18, whereas its isomer, abnormal cannabidiol, is a full GPR18 agonist with higher potency.¹¹ Like the CB receptor subtypes CB₁ and CB₂, GPR18 is coupled to G_i proteins mediating an inhibition of adenylate cyclase.¹² Furthermore, mitogen-activated protein (MAP) kinase activation was reported.¹³ So far, very little is known about the (patho)physiological roles of GPR18. Studies have shown that it is highly expressed in testes, spleen, endometrium and metastatic melanoma cells, and at lower levels in thymus and in peripheral white blood cells.^{8,13,14} Due to its distribution GPR18 is thought to be involved in maintaining tumor cell survival, *e.g.* of melanoma cells, and to modulate immune functions.^{8,14} In addition, activation of GPR18 was reported to induce migration of human endometrial cells.¹⁰ Thus GPR18 antagonists may be useful novel therapeutics for the treatment of endometriosis and cancer.

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† Electronic supplementary information (ESI) available: Screening results for agonistic activity in β-arrestin recruitment assays (GPR18 and GPR55). *K_i* values for the compounds at rat CB₁ receptors determined in rat brain cortical membrane preparations. Results from GTPγS binding studies of selected compounds at native rat cannabinoid CB₁ receptors and human cannabinoid CB₁ receptors. Effects of selected compounds on forskolin-induced cAMP accumulation performed with CHO cells stably expressing human CB₁ or CB₂ receptors. Screening results of selected compounds at GPR35 and the benzodiazepine binding site of GABA_A receptors. See DOI: 10.1039/c3md00394a

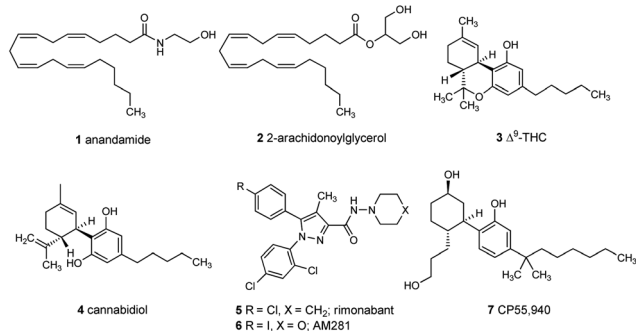


Fig. 1 Structures of selected cannabinoids and CB receptor ligands reported to interact with GPR18 and/or GPR55.

The orphan GPCR GPR55 is another receptor which has been found to be activated by certain CB receptor ligands, including the CB₁ inverse agonist rimonabant (5), and inhibited by others, such as the CB receptor agonist CP55,940 (7).^{7,15,16} GPR55 activation leads to G_{12/13} protein-mediated activation of RhoA, which in turn activates phospholipase C *via* Rho kinase (ROCK) resulting in a subsequent release of Ca²⁺ from intracellular stores.¹⁷ GPR55 is highly expressed in adrenal glands, the central nervous system, certain cancer cell lines and immune cells as well as osteoclasts and osteoblasts.^{16,18–20} The receptor has been postulated to play a role in mediating inflammatory and neuropathic pain, in promoting cancer cell migration and proliferation, and in the regulation of bone mass.^{18,21,22} GPR55 receptor antagonists are therefore considered as potential drug candidates for the treatment of cancer, neuropathic pain and osteoporosis.^{2,18,21,22}

Despite their interaction with several CB receptor ligands, GPR18 and GPR55 are not closely related to the classical CB receptors. Their reported endogenous agonists *N*-arachidonoylglycine (NAGly, 8, for GPR18), an anandamide metabolite, and 1-lyso-phosphatidylinositol (9, for GPR55) (Fig. 2) are inactive at CB₁ and CB₂ receptors.^{2,11,22–24} In addition, studies investigating an interaction of the endocannabinoids anandamide (1) and 2-arachidonoylglycerol (2) with GPR18 and GPR55 are inconsistent. McHugh *et al.* reported on activation of GPR18 using anandamide (1) and several research groups described anandamide (1) and 2-arachidonoylglycerol (2) as agonists at GPR55.^{11,23} But those results could not be confirmed by other groups.^{7,22,25,26} Furthermore, the degree of sequence identity between CB receptors and GPR18 (identity to CB₁ 12% and to CB₂ 7%), and CB receptors and GPR55 (identity to CB₁ 15% and

to CB₂ 13%) is relatively low, compared to an identity of 44% between both CB receptor subtypes.

The development of potent and selective ligands for GPR18 and GPR55 is required to further study the receptors' role in health and disease, and to explore their potential as drug targets.^{15,22} In particular, antagonists for GPR18 and GPR55 are urgently needed research tools. So far only two studies have reported the development of selective GPR55 receptor antagonists,^{27,28} and to the best of our knowledge no selective GPR18 antagonists have so far been described. In addition, it is important to investigate established and new CB receptor ligands for their potential interaction with GPR18 and GPR55, since some of the (adverse) effects of CB receptor ligands may be actually mediated by interaction with the orphan receptors GPR18 and/or GPR55.

Neither for the orphan GPCRs GPR18 and GPR55, nor for the CB receptors, crystal structures have been determined yet. Thus, screening campaigns, followed by structure–activity relationship (SAR) studies, currently appear to be the most promising approach to identify new ligands for the poorly characterized orphan receptors GPR18 and GPR55. Therefore we screened part of our proprietary compound library focusing on lipophilic structures (http://www.pharma.uni-bonn.de/www/pharmchem1/mueller-laboratory/compound-library?set_language=en). By performing β-arrestin translocation, and cAMP accumulation assays, respectively, we identified new lead structures for GPR18 and GPR55, as well as for CB₁ and CB₂ receptors, and subsequently optimized them for the new target receptors. Our study provides SARs of a series of 49 synthesized compounds at all four CB and CB-like receptors, and thus enables new insights into the structural requirements to selectively address each of these promising drug targets.

Results and discussion

Syntheses

The target compounds were synthesized starting from *Z*-5-arylidene-2-thiohydantoin, or from 5,5-diphenyl-2-thiohydantoin, respectively, by previously developed methods.^{29–38} 5-Arylidene-2-thiohydantoin derivatives were obtained by Knoevenagel condensation of aldehydes or ketones with 2-thiohydantoin (methods A–C).

Cyclization of 2-thiohydantoin derivatives with dibromopropane or dibromobutane under phase-transfer catalysis in acetone, in the presence of potassium carbonate and benzyltriethylammonium chloride (BTEA) as a catalyst, provided the desired *Z*-7-arylidene-imidazo[2,1-*b*]thiazines 10–39 and the *Z*-8-arylidene-imidazo[2,1-*b*]thiazepines 40–50. *Z*-7-Arylidene-imidazo[2,1-*b*]thiazines were prepared by 1,2- (39) or 2,3- (10–38) cyclization of the starting 2-thiohydantoin derivatives (Scheme 1). All described arylidene derivatives were obtained as *Z*-configuration stereoisomers as confirmed for several examples by X-ray structure analysis. According to our extensive experience with this class of compounds, the arylidene double bond is not reactive towards nucleophiles, which can be explained by its conjugation with the ring system.

5,5-Diphenyl-2-thiohydantoin was alkylated with dibromoalkanes (dibromoethane for 51 and 53, dibromopropane for

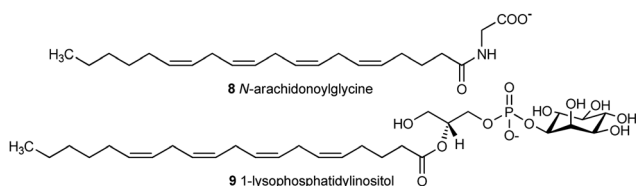
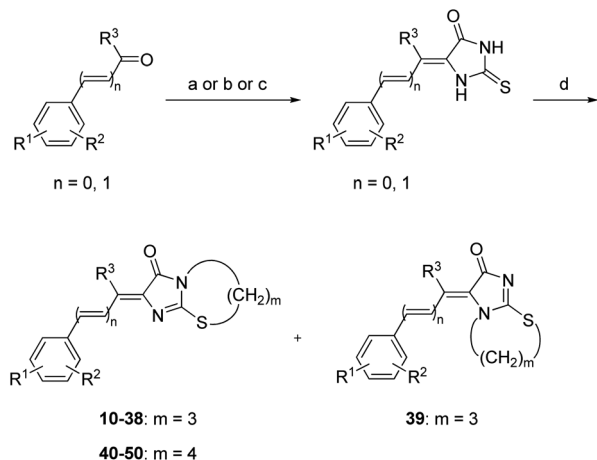
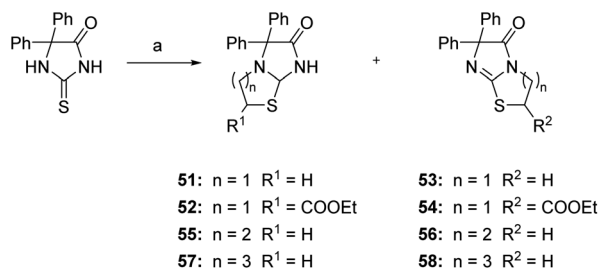


Fig. 2 Postulated endogenous agonists at GPR18 (NAGly, 8)¹³ and GPR55 (LPI, 9).²²



Scheme 1 Synthesis of arylidene-imidazo[2,1b]thiazine and -imidazo[2,1b]thiazepine derivatives. *Reagents and conditions:* (a) 2-thiohydantoin, alanine, Na_2CO_3 , water, reflux; (b) 2-thiohydantoin, sodium acetate, acetic acid, reflux; (c) 2-thiohydantoin, ammonium acetate, toluene, reflux; and (d) $\text{Br}(\text{CH}_2)_m\text{Br}$, BTEA, K_2CO_3 , acetone, rt.



Scheme 2 Synthesis of diphenyl-imidazo[2,1-b]-thiazole, -imidazo[2,1-b]thiazine and -imidazo[2,1-b]thiazepine derivatives. *Reagents and conditions:* (a) $\text{BrCH}_2\text{CH}(\text{Br})\text{COOEt}$ or $\text{Br}(\text{CH}_2)_n\text{Br}$, BTEA, K_2CO_3 , acetone, rt.

55 and **56**, and dibromobutane for **57** and **58**, or ethyl 2,3-dibromopropionate for **52** and **54**) to yield the products of 1,2- (**51**, **52**, **55**, **57**) or 2,3- (**53**, **54**, **56**, **58**) cyclization as depicted in Scheme 2.

The obtained compounds were purified by column chromatography. Their structures were confirmed by spectral analyses including IR, ^1H -NMR, ^{13}C NMR, and ESI-MS spectra. Purity was confirmed by TLC, LC-MS and elemental analyses (for details see Experimental section and ESI†).

Biological evaluation

Imidazo[2,1-*b*]thiazole (**51**, **52**, **53**, **54**), imidazo[2,1-*b*]thiazine (**10-39**, **55**, **56**) and imidazo[2,1-*b*]thiazepine (**57**, **58**, **40-50**) derivatives were investigated in order to assess their potential to interact with the orphan receptors GPR18 and GPR55, as well as with the two established CB receptor subtypes CB_1 and CB_2 , and to study their SARs. Due to the lack of potent radioligands for the orphan receptors GPR18 and GPR55 interaction of the compounds with these receptors was determined in β -arrestin translocation assays based on the β -galactosidase enzyme fragment complementation technology (β -arrestin

PathHunter™ assay, DiscoverX, Fremont, CA, USA). This assay format has been shown to be very useful for the investigation of orphan receptors.^{26,28,39-41} The measured luminescence signal is specific for the investigated receptor in a recombinant cell line (Chinese hamster ovary cells) stably expressing the respective human GPCR, since artefacts due to activation of endogenous receptors can be excluded.²⁶ For additional confirmation of signal specificity, a small subset of compounds was additionally tested at the human GPR35 using the same technology. The compounds were initially screened for agonistic and antagonistic activity at a concentration of 10 μM . Screening for antagonistic activity was performed by preincubation of the cells with the test compound followed by stimulation with a standard agonist (GPR18: Δ^9 -THC, 10 μM , corresponding to its EC_{90} ; GPR55: LPI, 1 μM , corresponding to its EC_{50} ; GPR35: zaprinast, 5 μM , corresponding to its EC_{80}).^{39,42} IC_{50} and/or K_{B} values were determined for compounds showing an inhibition of >60% of the effect produced by the standard agonist under the conditions described above. To investigate the affinity of the compounds for CB receptors, radioligand binding assays were performed at recombinant human CB_1 and CB_2 receptors stably expressed in CHO, or HEK293 (human embryonic kidney) cells, respectively. Most of the compounds were additionally tested at native rat CB_1 receptors in rat brain cortical membrane preparations. [^3H](−)-*cis*-3-[2-Hydroxy-4-(1,1-dimethyl-heptyl)-phenyl]-*trans*-4-(3-hydroxy-propyl)-cyclohexanol ([^3H]CP55,940, 7) was utilized as a CB_1 and CB_2 radioligand. Initially, the compounds were screened at a concentration of 10 μM . In cases where inhibition of radioligand binding was greater than 50%, full concentration–inhibition curves were determined in order to calculate K_{i} values. The most potent compounds of the present series were investigated for their functional properties in cAMP accumulation assays using CHO cells stably expressing the human CB_1 or CB_2 receptor subtype. In addition, [^{35}S]GTP γ S binding assays were conducted as a measure of receptor activation at rat brain cortical membranes, and at HEK cells stably expressing the human CB_1 , or CB_2 receptor subtype, respectively. Since some members of the investigated class of compounds had previously been found to interact with the benzodiazepine binding site of rat brain GABA $_A$ receptors²⁹ we performed binding studies for selected potent compounds *versus* [^3H]diazepam as previously described²⁹ to assess their selectivity (see the ESI, Table S3†).

Structure–activity relationships

Antagonistic potencies of the compounds at GPR18 and GPR55 are collected in Table 1, along with their binding affinities for CB_1 and CB_2 receptors. For direct comparison, Table 1 also contains test results of standard ligands obtained in our laboratory under the same conditions. These include Δ^9 -THC (**3**, (partial) agonist at GPR18, CB_1 and CB_2) *N*-arachidonoylglycine (NAGly, **8**, reported GPR18 agonist), LPI (**9**, agonist at GPR55), rimonabant (**5**, antagonist at GPR18; agonist at GPR55; inverse agonist at CB_1), and CP55,940 (**7**, antagonist at GPR18 and GPR55, agonist at CB_1 and CB_2).^{10,12,13,15,16} Comparison of IC_{50} values obtained from β -arrestin assays (GPR18, GPR55) with K_{i}

Table 1 Affinities and potencies of annelated imidazolone derivatives for GPR18, GPR55, CB₁ and CB₂ receptors^a

		β -Arrestin recruitment assay		Radioligand binding assays vs. [³ H]CP55,940	
		Human GPR18 IC ₅₀ ± SEM ^a (μM) (% of Δ ⁹ -THC inhibition) ^b	Human GPR55 IC ₅₀ ± SEM ^a (μM) (% of LPI inhibition) ^c	Human CB ₁ K _i ± SEM ^a (μM) (% inhibition) ^d	Human CB ₂ K _i ± SEM ^a (μM) (% inhibition) ^d
Compound	R				
Standard ligands					
3	See Fig. 1	Agonist EC ₅₀ 4.61 (ref. 28)	14.2 (ref. 28)	0.00388 ± 0.00091	0.0716 ± 0.0024
5	See Fig. 1	10.1 (ref. 28)	Agonist EC ₅₀ 2.01 (ref. 40)	0.0126 (ref. 44)	0.900 (ref. 44)
7	See Fig. 1	5.99 (ref. 28)	1.61 (ref. 40)	0.00128 (ref. 44)	0.00142 (ref. 44)
8	See Fig. 2	>10 (0%)	>10 (6%)	>10 (43%)	>10 (36%)
9	See Fig. 2	>10 (15%)	Agonist EC ₅₀ 1.00 (ref. 40)	>10 (0%)	>10 (0%)
Imidazo[2,1-<i>b</i>][1,3]thiazin-3-ones (A)					
10		>10 (5%)	>10 (25%)	>10 (40%)	>10 (18%)
11		>10 (18%)	>10 (35%)	>10 (25%)	>10 (23%)
12		>10 (14%)	≥10 (42%)	>10 (30%)	>10 (2%)
13		>10 (6%)	5.09 ± 0.35	>10 (0%)	>10 (21%)
14		>10 (0%)	6.91 ± 1.04	>10 (12%)	>10 (40%)
15		>10 (14%)	6.76 ± 1.37	>10 (8%)	>10 (24%)
16		>10 (0%)	>10 (0%)	>10 (14%)	>10 (4%)

Table 1 (Contd.)

		β -Arrestin recruitment assay		Radioligand binding assays vs. [3 H]CP55,940	
		Human GPR18 IC ₅₀ \pm SEM ^a (μ M) (% of Δ^9 -THC inhibition) ^b	Human GPR55 IC ₅₀ \pm SEM ^a (μ M) (% of LPI inhibition) ^c	Human CB ₁ K _i \pm SEM ^a (μ M) (% inhibition) ^d	Human CB ₂ K _i \pm SEM ^a (μ M) (% inhibition) ^d
Compound	R				
17		>10 (17%)	5.56 \pm 1.10	>10 (13%)	>10 (0%)
18		>10 (3%)	3.15 \pm 0.20	>10 (7%)	>10 (0%)
19		>10 (6%)	3.46 \pm 1.34	>10 (13%)	>10 (0%)
20		>10 (13%)	>10 (16%)	>10 (29%)	>10 (8%)
21		>10 (20%)	>10 (12%)	>10 (0%)	>10 (20%)
22		>10 (6%)	>10 (20%)	>10 (19%)	>10 (10%)
23		>10 (10%)	>10 (16%)	>10 (39%)	>10 (6%)
24		>10 (0%)	>10 (33%)	>10 (4%)	>10 (27%)
25		>10 (2%)	>10 (12%)	>10 (7%)	>10 (18%)
26		>10 (10%)	16.4 \pm 5.8	>10 (18%)	>10 (15%)

Table 1 (Contd.)

		β -Arrestin recruitment assay		Radioligand binding assays vs. [3 H]CP55,940	
		Human GPR18 $IC_{50} \pm SEM^a$ (μM) (% of Δ^9 -THC inhibition) b	Human GPR55 $IC_{50} \pm SEM^a$ (μM) (% of LPI inhibition) c	Human CB ₁ $K_i \pm SEM^d$ (μM) (% inhibition) d	Human CB ₂ $K_i \pm SEM^d$ (μM) (% inhibition) d
Compound	R				
27		>10 (7%)	9.77 ± 0.34	≥ 10 (42%)	2.57 ± 1.09
28		>10 (4%)	>10 (38%)	>10 (12%)	>10 (17%)
29		>10 (13%)	>10 (13%)	>10 (29%)	16.6 ± 6.2
30		~ 10 (54%)	>10 (33%)	>10 (29%)	>10 (9%)
31		7.22 ± 3.42	>10 (0%)	>10 (25%)	1.11 ± 0.01
32		0.279 ± 0.111	>10 (0%)	>10 (0%)	4.03 ± 0.51
33		1.65 ± 0.45	≥ 10 (45%)	>10 (0%)	>10 (9%)
34		2.55 ± 0.71	>10 (0%)	2.09 ± 0.08	0.95 ± 0.27
35		6.16 ± 2.86	11.8 ± 2.1	>10 (15%)	>10 (0%)
36		6.35 ± 1.86	>10 (7%)	>10 (2%)	≥ 10 (47%)

Table 1 (Contd.)

		β -Arrestin recruitment assay		Radioligand binding assays vs. [3 H]CP55,940	
		Human GPR18 IC ₅₀ \pm SEM ^a (μ M) (% of Δ^9 -THC inhibition) ^b	Human GPR55 IC ₅₀ \pm SEM ^a (μ M) (% of LPI inhibition) ^c	Human CB ₁ K _i \pm SEM ^a (μ M) (% inhibition) ^d	Human CB ₂ K _i \pm SEM ^a (μ M) (% inhibition) ^d
Compound	R				
37		14.3 \pm 3.26	>10 (16%)	>10 (12%)	>10 (0%)
38		>10 (0%)	>10 (27%)	>10 (16%)	>10 (37%)
Imidazo[2,1-b][1,3]thiazin-2-ones (B)					
39		>10 (2%)	>10 (0%)	>10 (26%)	>10 (19%)
Imidazo[2,1-b][1,3]thiazepin-3-ones (C)					
40		>10 (37%)	>10 (0%)	>10 (-5%)	5.43 \pm 1.31
41		14.1 \pm 2.4	>10 (33%)	1.60 \pm 0.42	>10 (21%)
42		~10 (49%)	>10 (21%)	2.13 \pm 0.47	1.81 \pm 0.34
43		>10 (31%)	Agonist (EC ₅₀) 10.7 \pm 0.3	0.25 \pm 0.06	4.15 \pm 0.39
44		>10 (15%)	>10 (27%)	2.16 \pm 0.07	>10 (37%)
45		6.48 \pm 1.20	\geq 10 (43%)	2.29 \pm 0.46	0.98 \pm 0.23

Table 1 (Contd.)

		β -Arrestin recruitment assay		Radioligand binding assays vs. [3 H]CP55,940	
		Human GPR18 IC ₅₀ \pm SEM ^a (μ M) (% of Δ^9 -THC inhibition) ^b	Human GPR55 IC ₅₀ \pm SEM ^a (μ M) (% of LPI inhibition) ^c	Human CB ₁ K _i \pm SEM ^d (μ M) (% inhibition) ^d	Human CB ₂ K _i \pm SEM ^d (μ M) (% inhibition) ^d
Compound	R				
46		4.61 \pm 2.36	>10 (33%)	3.18 \pm 0.49	1.65 \pm 0.71
47		>10 (5%)	>10 (12%)	0.85 \pm 0.03	1.56 \pm 0.34
48		>10 (22%)	>10 (4%)	4.78 \pm 1.70	>10 (39%)
49		6.45 \pm 1.10	>10 (32%)	>10 (35%)	2.58 \pm 1.45
Imidazo[2,1-b][1,3]thiazepin-3-ones (D)					
50	See above for structure	>10 (4%)	>10 (25%)	>10 (10%)	>10 (17%)
Imidazo[2,1-b]thiazol-6-ones (E, n = 1)					
51	-H	>10 (0%)	>10 (31%)	>10 (30%)	>10 (6%)
52	-COOC ₂ H ₅	>10 (0%)	>10 (41%)	>10 (26%)	>10 (-5%)
Imidazo[2,1-b]thiazol-5-ones (F, n = 1)					
53	-H	>10 (12%)	>10 (39%)	21.1 \pm 4.51	>10 (3%)
54	-COOC ₂ H ₅	>10 (0%)	>10 (10%)	>10 (2%)	>10 (0%)
Imidazo[2,1-b][1,3]thiazin-2-ones (E, n = 2)					
55	-H	>10 (0%)	>10 (2%)	>10 (13%)	>10 (9%)
Imidazo[2,1-b][1,3]thiazin-3-ones (F, n = 2)					
56	-H	>10 (0%)	>10 (23%)	>10 (25%)	>10 (5%)
Imidazo[2,1-b][1,3]thiazepin-2-ones (E, n = 3)					
57	-H	>10 (0%)	>10 (32%)	>10 (34%)	>10 (10%)
Imidazo[2,1-b][1,3]thiazepin-3-ones (F, n = 3)					
58	-H	>10 (0%)	>10 (6%)	1.34 \pm 0.37	>10 (14%)

^a All data result from three independent experiments, performed in duplicate. ^b Δ^9 -THC was used at a concentration of 10 μ M in preliminary screening assays, and at a concentration of 7.5 μ M for the determination of IC₅₀ values. ^c LPI was used at a concentration of 1 μ M.

^d Percent inhibition of [3 H]CP55,940 binding (0.1 nM) by test compounds at a concentration of 10 μ M.

values from radioligand binding studies (CB₁, CB₂) will provide a rough estimate of the compounds' selectivity.

We could confirm that Δ^9 -THC (**3**) behaves as a GPR18 agonist in β -arrestin recruitment assays.²⁸ The obtained EC₅₀ value of 4.61 μ M is consistent with literature data (EC₅₀ of 0.96 μ M) determined in a MAP kinase assay in HEK293 cells stably expressing GPR18.¹¹ However, the second reported GPR18 agonist, NAGly (**8**), failed to activate GPR18 in our test system. Our results support the recent findings of two other groups which also failed to observe activation of GPR18 by NAGly in several assay systems.^{26,27} Thus, further investigations are required to reveal the true role of NAGly in GPR18-mediated signalling. The non-selective CB agonist CP55,940 (**7**) and the CB₁-selective inverse agonist rimonabant (**5**) displayed antagonistic activities at GPR18 with potencies in the low micromolar concentration range.²⁸ In agreement with literature data, we could confirm that CP55,940 (**7**) and rimonabant (**5**) also interact with GPR55,^{7,15,16,40} rimonabant (**5**) being an agonist (EC₅₀ 2.01 μ M), while CP55,940 (**7**) was shown to be an antagonist at GPR55 (IC₅₀ 1.61 μ M).⁴⁰ Like **7**, Δ^9 -THC also behaved as an antagonist at GPR55.²⁸ The determined affinities of the standard cannabinoids for CB receptors are consistent with published data.⁴³

In the newly investigated series of bicyclic imidazole-4-ones, compounds with selectivity for either GPR18 or GPR55, or for CB₁ receptors, respectively, were discovered. Almost all of the potent compounds behaved as antagonists or inverse agonists at the respective receptor. The imidazo[2,1-*b*]thiazine (**A**) and the imidazo[2,1-*b*]thiazepine (**C**) scaffolds were found to be suitable for the development of GPR18 antagonists, imidazo[2,1-*b*]thiazines (**A**) generally displaying higher potency as compared to the analogous imidazo[2,1-*b*]thiazepines (**C**) (compare **31** and **42**, **32** and **43**, **33** and **45**, **34** and **46**). The potency of the compounds at GPR18 correlated with the size of the residues in position 2 of the annelated heterocycle: while *Z*-benzylidene-substituted imidazo[2,1-*b*]thiazines (**R** = (substituted) phenyl, **10–29**) failed to interact with GPR18, bulky biaromatic residues such as *m*-benzyloxybenzylidene (**31**, GPR18 IC₅₀ 7.22 μ M) and *m*-benzoyloxybenzylidene (**36**, GPR18 IC₅₀ 6.35 μ M) led to a remarkable increase in inhibitory potency. Introduction of chlorine atoms at the benzyloxybenzylidene residue further enhanced potency of the compounds, especially in the imidazothiazine series **A** (compare **31** with **32–35**) and in some cases had a great impact on selectivity. Introduction of a *p*-chloro-substituted benzyloxy residue (**32**), for instance, shifted the affinity towards GPR18 (GPR18 IC₅₀ 0.279 μ M, no interaction with GPR55 and CB₁, *K*_i CB₂ 4.03 μ M), while an *o,p*-dichloro-substituted benzyloxy moiety (**34**) enhanced affinity for CB₁ and CB₂ (GPR18 IC₅₀ 2.55 μ M, *K*_i CB₁ 2.09 μ M, *K*_i CB₂ 0.95 μ M, no interaction with GPR55). *o*-Chloro- (**33**) and *m,p*-dichloro- (**35**) substituted benzyloxy-substituted derivatives displayed no affinity for CB receptors, but considerable potency at GPR18 (**33**, GPR18 IC₅₀ 1.65 μ M; **35**, GPR18 IC₅₀ 6.16 μ M). Although the *o*-chloro- (**33**, GPR18 IC₅₀ 1.65 μ M) and the *p*-chloro- (**32**, GPR18 IC₅₀ 0.279 μ M) derivatives were the most potent GPR18 antagonists of the present series, the combination realized in the *o,p*-dichloro derivative **34** was not additive (GPR18 IC₅₀ 2.55 μ M). Displacement of the (*p*-chloro)benzyloxy (**31**, **32**) by a (*p*-chloro)benzoyloxy (**36**, **37**) residue decreased affinity for CB₁

and CB₂ receptors, while the substitution was tolerated by GPR18, although reduced potency was observed in the case of the chlorinated compound **37**. However, it cannot be completely ruled out that the ester function might be partly hydrolyzed by esterases potentially present on the cell surface of the utilized CHO cells. The *p*-chlorobenzyloxybenzylidene-substituted imidazothiazine derivative **32** was the most potent GPR18 antagonist of the present series displaying an IC₅₀ value of 0.279 μ M, »36-fold selectivity over CB₁ and GPR55, and 14-fold selectivity over CB₂ receptors. In the imidazothiazepine series **C** only the introduction of an *ortho*- (**45**) but not a *para*-chloro substituent increased potency at GPR18 (**43**, compare to the unsubstituted benzyloxybenzylidene derivative **42**). If the imidazothiazinone scaffold was substituted by smaller fluoro-, chloro-, or methoxybenzylidene moieties instead of larger aromatic residues, potent GPR55 ligands could be obtained. Especially *ortho*-halogen (Cl, F) substitution led to an increase in inhibitory potency at GPR55. The most potent compounds of the series were the *o,m*-difluorobenzyldene derivative **18** (GPR55 IC₅₀ 3.15 μ M), the *o,p*-difluorobenzyldene derivative **19** (GPR55 IC₅₀ 3.46 μ M), and the *o*-chloro-benzylidene derivative **13** (GPR55 IC₅₀ 5.09 μ M), while the methoxy-substituted benzylidene derivatives were less potent at GPR55, the best one being the *o,p*-dimethoxybenzylidene derivative **27** (GPR55 IC₅₀ 9.77 μ M). As nearly all of the benzylidene-substituted imidazo[2,1-*b*]thiazines possessed no or only low affinities for GPR18 and CB receptors, they represent promising lead structures for the future development of more potent and selective GPR55 ligands. It appears that GPR55 prefers smaller ligands, while GPR18 as well as the CB receptors require larger, more lipophilic, lipid-like ligands, at least with respect to the present series of compounds.

Introduction of larger residues containing two aromatic rings as in imidazothiazines (**A**) **30–37** and in imidazothiazepines (**C**) **40–47** reduced the inhibitory potency of the compounds at GPR55. Except for **35** (IC₅₀ 11.8 μ M) none of the compounds of this subgroup displayed notable inhibition of GPR55. Our current findings are consistent with previous observations made in a different series of GPR55 antagonists based on a coumarin scaffold indicating that large, lipophilic residues were not required for high inhibitory potency at the LPI-activated GPR55, and selectivity *versus* the CB receptor subtypes including GPR18 can easily be reached.²⁸ Interestingly, the *p*-chlorobenzyloxybenzylidene-substituted imidazothiazepinone **43** behaved as a moderately potent agonist at GPR55 (EC₅₀ 10.7 μ M). However, **43** showed much higher affinity for CB receptors, especially for the CB₁ subtype (*K*_i CB₁ 0.25 μ M).

Compounds with notable CB receptor affinity were identified among those members of series **A** (**27**, **30–32**, **34**) and **C** (**40–47**, **49**), which contained large substituents mostly consisting of two connected aromatic rings, as well as in the diphenyl-substituted series **F** (**58**). It became evident that affinity of 5,5-diphenylimidazol-4-one derivatives (**51–58**) to CB receptors depended on the size of the heterocycle annelated to the imidazole ring. While imidazo-[2,1-*b*]thiazole derivatives (**51–54**) and the thiazine derivatives **55** and **56** displayed no or very low affinity to CB receptors, thiazepine derivatives, in particular **58**, showed increased affinity for the CB₁ receptor. Due to the synthetic

pathway two isomeric products were obtained: 1,2-substituted (series F) and 2,3-substituted imidazoles (series E).³⁴ The two isomeric series differed in their affinity for CB₁ receptors. The most potent compound of the 5,5-diphenylimidazol-4-one series, **58** (series F), containing a thiazepine ring showed a K_i value of 1.34 μ M at human CB₁ receptors and was selective versus the CB₂ receptor, GPR18 and GPR55. Displacement of the 5,5-diphenyl moiety by *Z*-benzylidene residues containing small substituents (**10–29**) or an (*E,E*)-phenylbutadienyl residue (**38** and **39**) generally reduced the affinity of the compounds for CB receptors. However, by introduction of large, lipophilic residues, such as unsubstituted or halogen-substituted *m*-benzyloxy-benzylidene residues (**31**, **32**, and **42–47**) CB receptor affinity was increased. Compounds with high CB receptor affinity, some of which showed selectivity for CB₁ (e.g. **43** K_i CB₁ 0.25 μ M, K_i CB₂ 4.15 μ M), could thus be obtained.

Most compounds were additionally tested in radioligand binding assays at native CB₁ receptors in rat brain cortical membrane preparations (see the ESI Table S1†). Species differences were found to be moderate, and apart from few exceptions K_i values were comparable in both species. Exceptions included **30–32** and **56** with somewhat higher affinity for rat than for human CB₁, and **45**, **48** with higher affinity for human than for rat CB₁ receptors (see Table S1†).

Our results revealed that compounds of the presented series with high affinity for CB receptors in most cases also interacted with GPR18, indicating that CB receptors and GPR18 require similar structural features for ligand interaction. An interaction with the established CB receptors as well as GPR18 required benzylidene residues with bulky, lipophilic substituents (**31**, **32**, **34**, **45**, **46**, **49**), while identified GPR55 ligands of the present series possessed benzylidene residues with comparably small substituents (**13–15**, **17–19**, **26–27**). Some compounds (e.g. **34** and **46**) blocked all three receptors, CB₁, CB₂, and GPR18 with similar potency. In particular the *o,p*-dichlorobenzoyloxybenzylidene-substituted imidazothiazine derivative **34** shows inhibition of CB₁ (K_i 2.09 μ M), CB₂ (K_i 0.95 μ M), and GPR18 (IC_{50} 2.55 μ M), and no measurable inhibition of GPR55 at 10 μ M.

Interestingly an increase in affinity of the presented compounds for GPR18 and for CB receptors generally resulted in a dramatic decrease in GPR55 potency, indicating that GPR55 differs considerably in the structural features required to address its binding pocket compared to the other receptors investigated in the present study. Unfortunately, homology modelling and ligand docking are still highly speculative for the investigated group of GPCRs since no X-ray structures are currently available for any of them or for any closely related member of the class A GPCR family. Although homology models have been published based on available, distantly related GPCRs,^{44–47} they do not yet appear suitable for explaining SARs and predicting optimized ligands, at least if not combined with mutagenesis studies.

In order to explore whether signals induced by the investigated compounds are specific, we tested a subset of compounds, including **10**, **13**, **18**, **27**, **32**, **43** and **44**, for their interaction (agonism or antagonism) with the GPR55-related orphan receptor GPR35. To allow for comparison we applied the same β -arrestin recruitment assay that had been used to

investigate interactions with GPR18 and GPR55. All compounds were tested for agonistic as well as antagonistic activity at a concentration of 10 μ M. Only compound **13** showed a moderate inhibitory effect at GPR35 (49% inhibition at 10 μ M), whereas none of the other tested compounds showed any activity at the human GPR35 (see the ESI, Table S2†). For example, **18** (IC_{50} at GPR55 3.15 μ M) was selective for GPR55 and it neither interacted with GPR18 nor with GPR35 in the same test system (β -arrestin translocation using the β -galactosidase complementation assay). Also, the most potent GPR18 antagonist of the present series, compound **32**, was totally inactive at GPR35. Therefore, a specific mode of action for the presented series at the investigated GPCRs can be assumed.

Selected compounds (**12**, **13**, **18**, **19**, **32**, **34**, **36**, **43**, and **46**) were additionally investigated for their interaction with the benzodiazepine binding site of GABA_A receptors at a test concentration of 10 μ M (see the ESI, Table S3†). Only compound **13** showed significant affinity (K_i 4.15 μ M), while all other investigated compounds lacked interaction or showed only low affinity. Thus, their selectivity could be confirmed including those of the GPR55 antagonist **18** and the GPR18 antagonist **32**.

Functional properties of bicyclic imidazole-4-one derivatives at GPR18 and GPR55

All compounds of the presented series were not only screened for antagonistic but also for agonistic activity at GPR18 and GPR55 in β -arrestin assays (see the ESI, Table S3†). For GPR18 only antagonistic screening assays revealed hits, which were further investigated to determine IC_{50} values using various concentrations of the test compounds. The GPR18 agonist Δ^9 -THC was used at a fixed concentration of 7.5 μ M and inhibition of the agonist-induced signal was observed. Nine potent GPR18 antagonists were identified, of which compound **32** was the most potent one, displaying an IC_{50} value of 0.279 μ M when tested versus 7.5 μ M THC (corresponding to the EC_{80} of THC) (Fig. 3A). As depicted in Fig. 3A the THC-effect could not be fully inhibited by **32**. This is most likely due to the moderate solubility of **32**, leading to precipitation at high concentrations. Another explanation could be that **32** is an allosteric antagonist

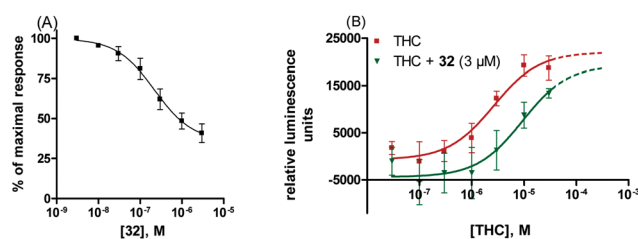


Fig. 3 (A) Concentration-dependent inhibition of Δ^9 -THC-induced β -arrestin recruitment to GPR18 by **32**. Δ^9 -THC was used at a concentration of 7.5 μ M. An IC_{50} value of 0.279 ± 0.111 μ M was determined for **32**. (B) Concentration-dependent LPI response in the absence (EC_{50} 2.11 ± 0.12 μ M) and in the presence of **32** (3 μ M) (EC_{50} 8.54 ± 0.71). A pA_2 value of 1.03 ± 0.17 μ M was calculated. Data are expressed as means \pm SEM of four separate experiments performed in duplicate.

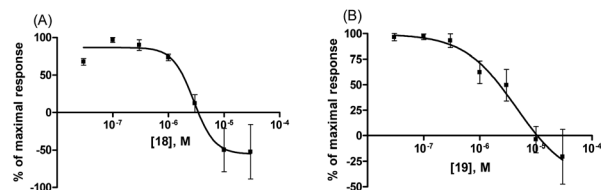


Fig. 4 Concentration-dependent inhibition of LPI- (1 μ M) induced GPR55-mediated β -arrestin recruitment by (A) **18** (IC_{50} , 3.15 ± 0.20 μ M) and (B) **19** (IC_{50} , 3.46 ± 1.34 μ M). Data represent means of three independent experiments performed in duplicate.

at GPR18. The inhibitory potency of compound **32** was further confirmed in assays determining concentration–response curves of the agonist Δ^9 -THC in the absence and in the presence of a fixed concentration of **32** (3 μ M, Fig. 3B). Antagonist **32** induced a parallel rightward shift of the concentration–response curve of THC, and a pA_2 value of 1.03 μ M could be determined, which was in the same range as its IC_{50} value, indicating a competitive mechanism of action.

Among the compounds that were active at the human GPR55, one agonist (**43**) as well as antagonists such as the selective compounds **18** (IC_{50} 3.15 μ M, Fig. 4A) and **19** (IC_{50} 3.46 μ M, Fig. 4B) were identified. Compounds **18** and **19** completely inhibited LPI- (1 μ M) induced receptor activation. At high concentrations of 10 μ M and 30 μ M, respectively, **18** and **19** decreased receptor activity even below basal values, indicating inverse agonism. Another explanation would be that the orphan GPR55 is already activated to a certain extent by natural agonists that might be present in the assay solution causing basal activation.

Recently published studies indicated that an allosteric binding site for cannabinoid-related compounds may be present on GPR55.^{23,28} To investigate whether the identified antagonists of the present series are targeting an allosteric binding pocket different from the LPI binding site, we performed concentration–response curves for the agonist LPI in the absence and presence of its antagonist (10 μ M). The results for GPR55 antagonists **13**,

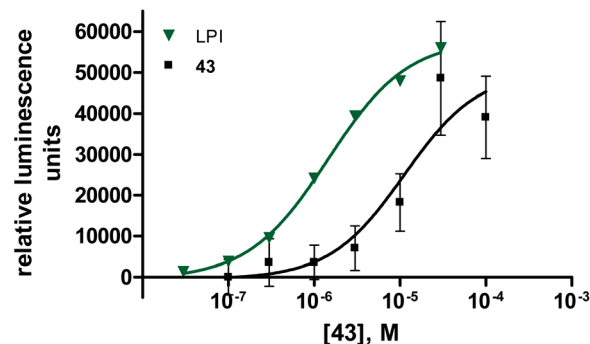


Fig. 6 Concentration-dependent β -arrestin recruitment to GPR55 induced by **43** (filled squares) (EC_{50} 10.7 ± 0.3 μ M). **43** exhibited comparable efficacy to LPI (green triangles) and was therefore characterized as a full agonist. Data are expressed as means \pm SEM of three separate experiments performed in duplicate.

14, **18** and **19** are presented in Fig. 5. At a high concentration of 10 μ M all compounds led to a rightward shift and at the same time to a remarkable depression of the maximal effect of the agonist–response curve, thus indicating allosteric antagonism. In addition, all compounds led to a reduction of basal receptor activity, which is an indication that they may be antagonists with inverse agonistic activity. Thus, the investigated compounds may be characterized as allosteric inverse agonists. Compounds bearing chloro-substituted benzylidene residues (**13**, **14**) led to a stronger depression of agonist efficacy, but were less potent than the investigated fluoro-benzylidene-substituted compounds **18** and **19** (Fig. 5 and Table 1). Since the experiments require a long incubation time of 3.5 h for the antagonists (1 h of pre-incubation), and 2.5 h for the agonist, the observed results cannot be due to different dissociation kinetics of both ligands.²⁸ However, to clearly confirm an allosteric binding mode, dissociation kinetic studies would be required, which, however, cannot be performed at present due to the lack of an appropriate radiolabeled or fluorescent-labeled ligand.^{23,28}

Compound **43** is the only compound of the present series displaying significant agonistic activity at GPR55. Compared to LPI, **43** displayed similar efficacy, and can therefore be characterized as a full agonist (Fig. 6). Compound **43** is an imidazo[2,1-*b*]thiazepine substituted with a bulky *p*-chloro-benzyloxybenzylidene residue. Our results showed that imidazo[2,1-*b*]thiazines and imidazo[2,1-*b*]thiazepines containing large bulky substituents are generally not well tolerated by GPR55. (Formal) moving of the chlorine atom of **43** from the *para*- to the *meta*- (**44**) or *ortho*-position (**45**) abolished potency of the respective compounds for GPR55. These results indicate steep SARs of the investigated scaffolds regarding their interaction with GPR55. Comparable results had been reported for the diphenylpyrazole derivatives 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-1-piperidinyl-1*H*-pyrazole-3-carboxamide (AM251) and 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM281, **6**).¹⁶ The displacement of the piperidinyl residue in AM251 by a morpholinyl residue in **6** was shown to lead to a more than 20-fold reduction in

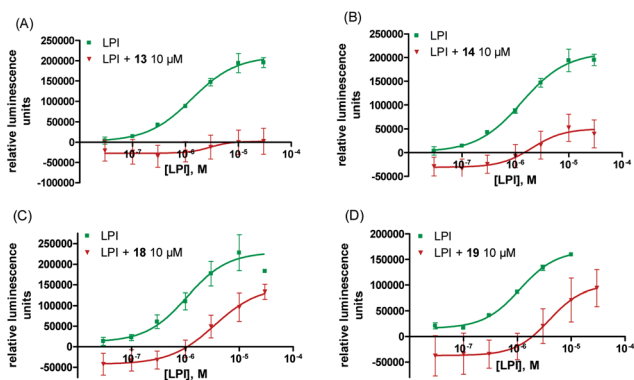


Fig. 5 Concentration–response curve of LPI in β -arrestin assays in the absence and presence (10 μ M) of (A) **13** (LPI EC_{50} 1.30 ± 0.18 μ M, LPI + **13** EC_{50} 17.7 ± 12.3 μ M), (B) **14** (LPI EC_{50} 1.12 ± 0.06 μ M, LPI + **14** EC_{50} 5.10 ± 2.72 μ M), (C) **18** (LPI EC_{50} 1.29 ± 0.17 μ M, LPI + **18** EC_{50} 2.40 ± 0.18 μ M) and (D) **19** (LPI EC_{50} 1.16 ± 0.07 μ M, LPI + **19** EC_{50} 3.07 ± 0.38 μ M) at CHO cells stably expressing the human GPR55. Data represent means of three independent experiments performed in duplicate.

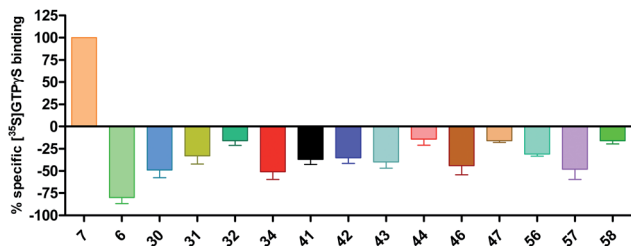


Fig. 7 [³⁵S]GTPγS binding assay at human CB₁ cannabinoid receptors expressed in human embryonic kidney (HEK293) cells. All compounds were tested at a concentration of 10 μM. Obtained data were normalized with respect to the maximal effect of 7 (10 μM, set at 100%). Data are expressed as means ± SEM of at least three separate experiments performed in triplicate.

activity at GPR55 (AM251 EC₅₀ 0.63 μM, 6 EC₅₀ 13.6 μM, calcium mobilization assay).¹⁶

Functional properties of bicyclic imidazol-4-one derivatives at cannabinoid receptors

In order to determine the intrinsic activity of the present series at CB receptors, we investigated the compounds with the highest affinity as determined in radioligand binding studies additionally for their function (agonistic, antagonistic, inverse agonistic activity). Therefore, [³⁵S]GTPγS binding assays were performed at human recombinant and at rat brain CB₁ receptors. GDP–GTP exchange is an early event in the signal transduction mechanism of a GPCR. Measuring the binding of [³⁵S]GTPγS – a radiolabeled nonhydrolyzable analogue of GTP – provides direct quantitative information on the interaction between the receptor and the associated G protein. Binding of an agonist to the GPCR will increase binding of the guanine nucleotide, while a neutral antagonist will have no effect, and an inverse agonist will decrease [³⁵S]GTPγS binding to the G protein. The compounds were tested at a concentration of 10 μM, and the determined effects were compared to those elicited by the full agonist 7 (10 μM) and the inverse agonist 6 (10 μM).

As expected, 7 induced an increase in [³⁵S]GTPγS binding from a basal level of 100% to 161% in membranes prepared from human embryonic kidney (HEK293) cells expressing the human CB₁ receptor, and to 132% at CB₁ receptors natively expressed in rat brain cortical membranes. The effects of the investigated compounds were normalized to the effect evoked by 7 (see Fig. 7 and Table 2 in the ESI†).

Antagonist 6, previously described as an inverse agonist,⁴⁸ led to a large reduction of GTPγS binding from 0 to –80% at human CB₁ receptors expressed in HEK293 cells. However, compound 6 had only a little effect on [³⁵S]GTPγS binding to natively expressed CB₁ receptors in rat brain cortex. The discrepancy in the efficacy of the inverse agonist 6 at native (rat) CB₁ receptors compared to (human) CB₁ receptors expressed in HEK293 cells is likely due to the higher density of CB₁ receptors in the overexpressing recombinant cell system as compared to native brain tissue. Similar effects had been observed before *e.g.* for the antagonist/inverse agonist 5.^{49,50}

None of the investigated compounds led to an increase in GTPγS binding at rat brain CB₁ receptors, thus showing that they were all antagonists (see the ESI, Table S4†).

In HEK293 cells, which were stably transfected with the human CB₁ receptor, the selected imidazol-4-one derivatives induced a decrease in basal [³⁵S]GTPγS binding by 14% to 51%, indicating inverse agonistic activity of the compounds in a test system with receptor overexpression. Compounds 30, 34, 43, 46 and 57 displayed the greatest negative intrinsic activity (up to approximately –50%). The investigated compounds showed in most cases a good correlation between their affinity and the effects observed in GTPγS binding studies, with only a few exceptions. The *p*-fluorobenzyloxybenzylidene-substituted imidazo[2,1-*b*]thiazepine derivative (47) showed high CB₁ receptor affinity, but showed only a low degree of inverse agonistic activity when compared to the unsubstituted benzyl-oxybenzylidene analogue 42. The *m*-chlorobenzyloxybenzylidene derivative (44) displayed similarly low intrinsic activity to 47. Compound 57, a 5,5-diphenylimidazo[2,1-*b*]thiazepine, exhibited an approximately 5-fold decreased affinity to the rat CB₁ receptor compared to its 6,6-diphenyl-imidazo[2,1-*b*]thiazepine analogue 58, but 57 had a greater negative intrinsic activity (–48% (57) compared to –16% (58)), indicating that the position of the annelated sulfur-containing heterocycle may have an impact on the efficacy of the compounds. Further GTPγS binding studies showed that compound 43 displayed inverse agonistic activity also at the human recombinant CB₂ receptor subtype where it decreased basal [³⁵S]GTPγS binding (see the ESI, Table S4†).

Finally, the most potent CB₁ (34, 43, 47, 58) and CB₂ antagonists (31, 34, 42, 45, 46, 47) of the present series were additionally investigated for potential effects on forskolin-induced cAMP accumulation in CHO-K1 cells stably expressing either human CB₁ or CB₂ receptors (see the ESI, Fig. S1 and S2†). In contrast to the agonist 7, none of the test compounds showed any effect at a high concentration of 10 μM indicating that they are receptor antagonists, and thus confirming the results obtained in GTPγS binding studies.

Conclusions

Imidazo[2,1-*b*]thiazole (51–54), imidazo[2,1-*b*]thiazine (10–39, 55–56), and imidazo[2,1-*b*]thiazepine derivatives (57–58, 40–50) were discovered as new scaffolds for the development of antagonists for the orphan GPCRs GPR18 and GPR55, and for the CB receptor subtypes CB₁ and CB₂. Introduction of a benzyloxybenzylidene residue (36, GPR18 IC₅₀ 6.35 μM, GPR55 and CB₁ K_i > 10 μM, CB₂ K_i ~ 10 μM) or a *p*-chlorobenzyloxybenzylidene residue (32, GPR18 IC₅₀ 0.279 μM, GPR55 and CB₁ K_i > 10 μM, CB₂ K_i 4.03 μM) into an imidazo[2,1-*b*]thiazine scaffold led to GPR18-selective antagonists. Compound 32 exhibited the highest inhibitory potency of all investigated compounds at GPR18 combined with »36-fold selectivity over CB₁ and 14-fold selectivity over CB₂ receptors. The compound did not interact with GPR55. To our knowledge, GPR18 antagonist 32 represents the first selective antagonist for this poorly investigated orphan receptor, which appears to have considerable potential as a novel drug target.

In contrast to CB₁, CB₂ and GPR18, our results indicate that GPR55 preferentially interacts with imidazo[2,1-*b*]thiazine derivatives possessing benzylidene residues without bulky substituents. The identified ligands of this subgroup, such as **18** and **19**, displayed antagonistic activity at GPR55 (**18** IC₅₀ 3.15 μM, **19** IC₅₀ 3.46 μM) and in general no affinity to CB receptors. The identified GPR55 antagonists did also not interact with GPR18. Thus, these new GPR55 antagonists represent suitable lead structures for the development of more potent, selective GPR55 antagonists. In addition to antagonists, we also identified one GPR55 receptor agonist (**43**, EC₅₀ 10.7 μM). Interestingly, **43** also displayed high affinity for CB receptors, but showed contrary intrinsic activity at CB receptors where it acted as an antagonist/inverse agonist. This phenomenon – reverse intrinsic activity at GPR55 *versus* CB receptors – had previously been reported for other CB receptor ligands, including **1**, **5** and **7**, and led to the designation of GPR55 as an ‘anti-cannabinoid receptor’.^{28,51} Compound **43** was the most potent ligand at CB₁ receptors and exhibited 17-fold selectivity over CB₂ receptors (*K*_i CB₁ 0.25 μM; CB₂ *K*_i 4.15 μM). The compound showed only a very weak inhibitory effect at GPR18.

In addition to selective antagonists for either of the investigated subtypes, compounds were identified which block GPR18 as well as CB₁ and CB₂ receptors at a similar concentration while not interacting with GPR55. The best triply active antagonist was **34** (GPR18 IC₅₀ 2.55 μM, CB₁ *K*_i 2.09 μM, CB₂, *K*_i 0.95 μM), which may become a useful research tool to study effects of the blockade of all three CB(-like) receptors.

This very first extensive SAR study of a series of 49 ligands investigated at all four CB and CB-like receptors reveals structural requirements for the development of potent and selective GPR18 and GPR55 antagonists. Selective GPR18 and GPR55 antagonists are crucial tools to pharmacologically characterize these orphan GPCRs, to explore their (patho-)physiological role and their potential as future drug targets.

Experimental section

Syntheses

Melting points were measured in open capillaries on a Mel-Temp. II apparatus (LD Inc., USA) and were not corrected. The purity of the synthesized compounds was confirmed by TLC performed on Merck silica gel 60 GF₂₅₄ aluminium sheets (Merck, Darmstadt, Germany), using the following developing system: CHCl₃ : AcOEt (1/1, v/v). Spots were detected by their absorption under UV light (λ = 254 nm). Column chromatography was performed on Merck silica gel 60 (70–230 mesh) using CH₂Cl₂ : AcOEt; 1/1 (v/v) as an eluent. Infrared spectra were measured with an FT IR 410 spectrometer (Jasco) in KBr pellets. ¹H-NMR spectra were obtained on a Varian Mercury spectrometer (Varian Inc., Palo Alto, CA, USA) in DMSO-*d*₆ or CDCl₃ operating at 300 MHz. Chemical shifts are reported in δ values (ppm) relative to undeuterated solvent signals. The *J* values are expressed in Hertz (Hz). Signal multiplicities are represented by the following abbreviations: s (singlet), d (doublet), dd (doublet), t (triplet), def.t (deformed triplet), m (multiplet). ¹³H-NMR spectra were acquired on a Varian Mercury spectrometer (Varian Inc., Palo Alto, CA, USA) in

CDCl₃, operating at 75 MHz. Mass spectrometry analyses: samples were prepared in a mixture of acetonitrile : water (50/50, v/v). The LC/MS system consisted of a Waters Acquity UPLC, coupled to a Waters TQD mass spectrometer (electrospray ionization mode ESI-tandem quadrupole). All analyses were carried out using an Acquity UPLC BEH C18, 100 × 2.1 mm reversed-phase column. A flow rate of 0.3 ml min⁻¹ and a gradient of 5–100% B over 10 min were used. Eluent A: water containing 0.1% HCO₂H; eluent B: acetonitrile containing 0.1% HCO₂H. Nitrogen was used as both nebulizing and drying gas. LC/MS data were obtained by scanning the first quadrupole in 0.5 s in a mass range from 50 to 1000 *m/z*; 10 scans were summed up to produce the final spectrum. Mass chromatograms of specific ions were recorded by tuning the first quadrupole on a targeted *m/z* value known as the single ion recording experiment. LC/MS/MS data were obtained under low energy collision induced dissociation (CID) conditions. The first quadrupole was fixed to the *m/z* value corresponding to the precursor ion to be dissociated, the collision cell (*q*) was filled with argon as collision gas and the last quadrupole was scanned within 0.5 s from 100 to 500 *m/z*; 10 scans were summed up to produce the final daughter ion mass spectra. Elemental analyses for C, H, N and S were carried out by a micro-method using the elemental Vario-EL III Elemental Analyser (Hanau, Germany) and the results were found to be within ±0.4% of the theoretical values. All tested compounds possessed a purity of not less than 95%.

Compounds **12**, **13**, **23**, **24**, **29**;¹ **11**, **21**;^{29,35} **10**, **20**, **22**;³² **57**, **58**;³⁶ **14**, **25**, **27**, **28**, **30**, **38**, **39**;³⁴ **56**;^{37,38} **52**, **54**;³⁰ **51**, **55**;³¹ and **40**, **41**, **43**, **46**, **48**, **49**, **50** (ref. 33) were prepared as described.

Preparation of imidazo[2,1-*b*][1,3]thiazinones **15**–**19**, **31**–**37** and imidazo[2,1-*b*][1,3]thiazepinones **42**, **44**, **45**, **47**

The new compounds **15**–**19**, **31**–**37**, **42**, **44**, **45** and **47** were prepared in analogy to described procedures.^{29,32} The general procedure was as follows:

To a stirred suspension of the appropriate (*Z*)-5-arylidene-2-thiohydantoin (0.01 mol), K₂CO₃ (0.029 mol) and benzyltriethylammonium chloride (0.001 mol) in 50 ml acetone, a solution of 1,3-dibromopropane (0.01 mol), or 1,4-dibromobutane (0.012 mol), respectively, in 10 ml acetone was added dropwise. The suspension was stirred for 48 h. Then the precipitate was filtered off, washed with water, then with 1% aq. NaOH, followed by 1% aq. HCl solutions (50 ml each). The remaining solid was suspended in chloroform (100 ml), stirred for 0.5 h, and the suspension was filtered. The solid (mp > 220 °C) was discarded. The chloroform filtrate was evaporated to dryness, the residue was dissolved in chloroform and washed with water, followed by 1% aq. NaOH solution (50 ml each), and then evaporated to dryness. The obtained solids or oily residues were purified by means of column chromatography.

Analytical data

(*Z*)-2-(2,4-Dichlorobenzylidene)-6,7-dihydro-2*H*-imidazo[2,1-*b*][1,3]thiazin-3(5*H*)-one (**15**). Product **15** was purified by crystallization; yellow powder, mp 225–228 °C (acetonitrile); yield 14.47%; ¹H-NMR (300 MHz; CDCl₃) δ: 2.24–2.32 (m, 2H, SCH₂CH₂),

3.15 (def.t, $J = 5.77$ Hz, 2H, SCH₂), 3.61 (t, $J = 5.90$ Hz, 2H, NCH₂), 7.25–7.29 (m, 1H, Ar H-5'), 7.31 (s, 1H, ArCH=), 7.41 (d, $J = 2.05$ Hz, 1H, Ar H-3'), 8.75 (d, $J = 7.84$ Hz, 1H, Ar H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ : 22.80 (CH₂); 26.20 (SCH₂); 39.86 (NCH₂); 117.43 (ArCH=); 127.43; 129.43; 130.74; 133.77; 135.62; 136.31; 138.50 (ArCH=C); 161.99 (C=O); 168.44 (C=N); IR ν : 1707 (C=O), 1629 (ArCH=), 1578 (C=N), 1481, 1462, 1362, 1282, 1261, 1165, 1117; $R_f = 0.61$; $t_R = 7.81$; purity 98.46%; MS calcd for [M + H]⁺: C₁₃H₁₀N₂OSCl₂ m/z : 313.20, found 313.21; anal. calcd: C 49.85, H 3.22, N 8.95, S 10.24; found: C 50.19, H 3.57, N 9.12, S 10.77%.

(Z)-2-(2,6-Dichlorobenzylidene)-6,7-dihydro-2H-imidazo[2,1-b]-[1,3]thiazin-3(5H)-one (16). Product 16 was purified by crystallization; cream yellow powder, mp 228–231 °C (acetonitrile); yield 41.9%; ¹H-NMR (300 MHz; CDCl₃) δ : 2.23–2.31 (m, 2H, SCH₂CH₂), 3.11 (def.t, $J = 5.64$ Hz, 2H, SCH₂), 3.74 (t, $J = 5.89$ Hz, 2H, NCH₂), 7.00 (s, 1H, ArCH=), 7.16–7.25 (m, 1H, Ar H-4'), 7.32 (d, $J = 8.46$ Hz, 2H, Ar H-3', Ar H-5'); ¹³C-NMR (75 MHz; CDCl₃) δ : 23.00 (CH₂); 26.11 (SCH₂); 39.97 (NCH₂); 119.16 (ArCH=); 128.17; 129.85; 132.00; 136.00; 141.00 (ArCH=C); 163.00 (C=O); 169.00 (C=N); IR ν : 1719 (C=O), 1644 (ArCH=), 1554 (C=N), 1494, 1471, 1425, 1392, 1329, 1245, 1164, 1121, 1093, 773; $R_f = 0.58$; $t_R = 5.90$; purity 99.56%; MS calcd for [M + H]⁺: C₁₃H₁₀N₂OSCl₂ m/z : 313.20, found 313.14; anal. calcd: C 49.85, H 3.22, N 8.95, S 10.24; found: C 50.03, H 3.42, N 8.91, S 10.71%.

(Z)-2-(2-Fluorobenzylidene)-6,7-dihydro-2H-imidazo[2,1-b]-[1,3]thiazin-3(5H)-one (17). Product 17 was purified by crystallization; yellow powder, mp 199–202 °C (acetonitrile); yield 12.7%; ¹H-NMR (300 MHz; CDCl₃) δ : 2.27–2.32 (m, 2H, SCH₂CH₂), 3.16 (def.t, $J = 5.64$ Hz, 2H, SCH₂), 3.75 (t, $J = 5.77$ Hz, 2H, NCH₂), 7.05 (dt, $J_1 = 9.29$ Hz, $J_2 = 1.16$ Hz, 1H, Ar H-3'), 7.17–7.22 (m, 1H, Ar H-4'); 7.27 (s, 1H, ArCH=), 7.29–7.36 (m, 1H, Ar H-5'); 8.74 (dt, $J_1 = 7.69$ Hz, $J_2 = 1.79$ Hz, 1H, Ar H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ : 22.87 (CH₂); 26.16 (SCH₂); 39.85 (NCH₂); 114.72 (ArCH=); 115.07; 122.49; 124.45; 131.41; 132.61; 138.16; 161.00 (ArCH=C); 163.40 (C=O); 168.60 (C=N); IR ν : 1704 (C=O), 1632 (ArCH=), 1552 (C=N), 1489, 1455, 1292, 1230, 1166, 1128, 762; $R_f = 0.62$; $t_R = 6.09$; purity 99.44%; MS calcd for [M + H]⁺: C₁₃H₁₁N₂OSF m/z : 262.30, found 263.28.

(Z)-2-(2,3-Difluorobenzylidene)-6,7-dihydro-2H-imidazo[2,1-b]-[1,3]thiazin-3(5H)-one (18). Product 18 was purified by crystallization; yellow powder, mp 227–230 °C (acetonitrile); yield 17.86%; ¹H-NMR (300 MHz; CDCl₃) δ : 2.25–2.33 (m, 2H, SCH₂CH₂), 3.17 (def.t, $J = 5.76$ Hz, 2H, SCH₂), 3.76 (t, $J = 5.90$ Hz, 2H, NCH₂), 7.08–7.16 (m, 2H, Ar H-4', Ar H-5'), 7.20 (s, 1H, ArCH=), 8.47–8.53 (m, 1H, Ar H-6'); 8.74 (dt, $J_1 = 7.69$ Hz, $J_2 = 1.79$ Hz, 1H, Ar H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ : 22.82 (CH₂); 26.18 (SCH₂); 39.87 (NCH₂); 113.12; 117.90 (ArCH=); 124.15; 124.53; 127.33; 139.00; 147.92; 151.31 (ArCH=C); 162.08 (C=O); 168.46 (C=N); $R_f = 0.64$; $t_R = 6.42$; purity 98.18%; MS calcd for [M + H]⁺: C₁₃H₁₀N₂OSF₂ m/z : 280.30, found 281.23.

(Z)-2-(2,4-Difluorobenzylidene)-6,7-dihydro-2H-imidazo[2,1-b]-[1,3]thiazin-3(5H)-one (19). Product (19) was purified by column chromatography and crystallization; yellow powder, mp 211–214 °C (acetonitrile); yield 11.9%; ¹H-NMR (300 MHz; CDCl₃) δ : 2.24–2.32 (m, 2H, SCH₂CH₂), 3.16 (def.t, $J = 5.64$ Hz, 2H, SCH₂), 3.75 (t, $J = 5.90$ Hz, 2H, NCH₂), 6.77–6.85 (m, 1H, Ar H-5'); 6.90–6.97 (m, 1H, Ar H-3'), 7.17 (s, 1H, ArCH=), 8.80 (dq, $J_1 = 6.92$

Hz, $J_2 = 1.92$ Hz, 1H, Ar H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ : 22.87 (CH₂); 26.15 (SCH₂); 39.85 (NCH₂); 103.37 (ArCH=); 111.85; 118.87; 133.84; 134.00; 137.93; 160.23; 161.83 (ArCH=C); 163.64 (C=O); 168.59 (C=N); $R_f = 0.68$; $t_R = 6.46$; purity 99.36%; MS calcd for [M + H]⁺: C₁₃H₁₀N₂OSF m/z : 280.30, found 281.23.

(Z)-2-(2,4-Dimethoxybenzylidene)-6,7-dihydro-2H-imidazo-[2,1-b][1,3]thiazin-3(5H)-one (26). Product 26 was purified by crystallization; light yellow powder, mp 199–202 °C (acetonitrile); yield 18.6%; ¹H-NMR (300 MHz; CDCl₃) δ : 2.23–2.30 (m, 2H, SCH₂CH₂), 3.13 (def.t, $J = 5.64$ Hz, 2H, SCH₂), 3.73 (t, $J = 5.90$ Hz, 2H, NCH₂), 3.86 (s, 3H, OCH₃), 3.87 (s, 3H, OCH₃), 6.91 (dd, $J_1 = 8.20$ Hz, $J_2 = 1.54$ Hz, 1H, Ar H-4'), 7.09 (t, $J = 8.33$ Hz, 1H, Ar H-5'), 7.43 (s, 1H, ArCH=), 8.30 (dd, $J_1 = 8.08$ Hz, $J_2 = 1.41$ Hz, 1H, Ar H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ : 22.92 (CH₂); 26.14 (SCH₂); 39.83 (NCH₂); 55.82 (OCH₃); 61.75 (OCH₃); 113.97 (ArCH=); 118.19; 124.15; 124.19; 128.40; 137.75; 149.28; 152.51 (ArCH=C); 159.98 (C=O); 168.87 (C=N); IR ν : 1708 (C=O), 1625 (ArCH=), 1573 (C=N), 1493, 1469, 1425, 1271, 1237, 1188, 1168, 1121, 1063, 997, 739; $R_f = 0.61$; $t_R = 5.68$; purity 95.09%; MS calcd for [M + H]⁺: C₁₅H₁₆N₂O₃S m/z : 304.36, found 305.23; anal. calcd: C 59.16, H 5.30, N 9.21, S 10.53; found: C 58.72, H 5.24, N 9.20, S 10.36%.

(Z)-2-(3-(Benzoyloxy)benzylidene)-6,7-dihydro-2H-imidazo-[2,1-b][1,3]thiazin-3(5H)-one (31). Product 31 was obtained after purification by column chromatography; yellow powder, mp 189–191 °C (dioxane); yield 15%; ¹H-NMR (300 MHz; DMSO-d₆) δ : 2.15–2.16 (m, 2H, SCH₂CH₂), 3.22 (def.t, $J = 5.47$ Hz, 2H, SCH₂), 3.61 (t, $J = 5.64$ Hz, 2H, NCH₂), 5.11 (s, 2H, CH₂O), 6.77 (s, 1H, ArCH=), 7.02 (dd, $J = 8.20$ Hz, $J = 1.80$ Hz, 1H, Ar H-4'), 7.29–7.41 (m, 4H, Ar H-5', Ar H-3'', H-4'', H-5''), 7.46 (dd, $J = 7.18$ Hz, $J = 1.60$ Hz, 2H, Ar H-2'', H-6''), 7.67 (d, $J = 7.60$ Hz, 1H, Ar H-6'), 7.86 (d, $J = 1.53$ Hz, 1H, Ar H-2''); ¹³C-NMR (75 MHz; CDCl₃) δ : 22.92 (CH₂); 26.11 (SCH₂); 39.85 (NCH₂); 70.05 (CH₂O); 117.10; 117.21; 124.06; 125.08; 127.74; 127.99; 128.58; 129.56; 135.50; 136.89; 137.52; 158.81 (ArCH=C); 160.02 (C=O); 168.96 (C=N); IR ν : 1709 (C=O), 1637 (ArCH=), 1569 (C=N), 1489, 1446, 1290, 1259, 1160, 1041, 747; $R_f = 0.49$; $t_R = 7.46$; purity 98.22%; MS calcd for [M + H]⁺: C₂₀H₁₈N₂O₂SC m/z : 350.42, found 351.2; anal. calcd: C 68.55, H 5.18, N 7.99, S 9.15; found: C 68.56, H 5.02, N 7.81, S 8.73%.

(Z)-2-(3-(4-Chlorobenzoyloxy)benzylidene)-6,7-dihydro-2H-imidazo-[2,1-b][1,3]thiazin-3(5H)-one (32). Product 32 was obtained by column chromatography; yellow powder, mp 189–191 °C; yield 12%; ¹H-NMR (300 MHz; DMSO-d₆) δ : 2.12–2.16 (m, 2H, SCH₂CH₂), 3.21 (def.t, $J = 5.51$ Hz, 2H, SCH₂), 3.60 (t, $J = 5.79$ Hz, 2H, NCH₂), 5.10 (s, 2H, CH₂O), 6.77 (s, 1H, ArCH=), 7.00 (dd, $J = 8.46$ Hz, $J = 2.05$ Hz, 1H, Ar H-4'), 7.32 (def.t, $J = 8.20$ Hz, 1H, Ar H-5'), 7.41–7.50 (m, 4H, Ar H-5'', H-3'', Ar H-2'', H-6''), 7.67 (d, $J = 7.95$ Hz, 1H, Ar H-6'), 7.85 (d, $J = 2.31$ Hz, 1H, Ar H-2''); ¹³C-NMR (75 MHz; CDCl₃) δ : 22.94 (CH₂); 26.12 (SCH₂); 39.85 (NCH₂); 69.22 (CH₂O); 116.84; 117.28; 123.87; 125.29; 128.73; 129.05; 129.58; 133.72; 135.44; 135.57; 137.67; 158.52 (ArCH=C); 160.04 (C=O); 168.97 (C=N); IR ν : 1703 (C=O), 1636 (ArCH=), 1578 (C=N), 1494, 1473, 1358, 1231, 1178, 1033, 797; $R_f = 0.49$; $t_R = 8.05$; purity 97.07%; MS calcd for [M + H]⁺: C₂₀H₁₇N₂O₂ClS m/z : 384.9, found 385.1; CC (CH₂Cl₂ : AcOEt

1 : 1); anal. calcd: C 62.41, H 4.45, N 7.28, S 8.33; found: C 62.22, H 4.58, N 7.10, S 8.51%.

(**Z**)-2-(3-(2-Chlorobenzyloxy)benzylidene)-6,7-dihydro-2*H*-imidazo[2,1-*b*][1,3]thiazin-3(5*H*)-one (33). Product 33 was purified by crystallization; yellow powder, mp 171–173 °C (twice crystallization, acetonitrile); yield 14.38%; ¹H-NMR (300 MHz; CDCl₃) δ: 2.23–2.30 (m, 2H, SCH₂CH₂), 3.13 (def.t, *J* = 5.64 Hz, 2H, SCH₂), 3.73 (t, *J* = 5.77 Hz, 2H, NCH₂), 5.22 (s, 2H, CH₂O), 6.92 (s, 1H, ArCH=), 6.98 (dd, *J* = 8.25 Hz, *J* = 0.77 Hz, 1H, Ar H-4'), 7.24–7.37 (m, 3H, Ar H-3'', Ar H-4'', H-6''), 7.41 (m, 1H, Ar H-5'), 7.56–7.59 (m, 1H, Ar H-6''); 7.64 (d, *J* = 7.69 Hz, 1H, Ar H-6'), 7.86 (t, *J* = 8.05 Hz, 1H, Ar H-2'); ¹³C-NMR (75 MHz; CDCl₃) δ: 22.93 (CH₂); 26.12 (SCH₂); 39.86 (NCH₂); 67.21 (CH₂O); 116.97; 117.36; 123.92; 125.22; 126.94; 128.94; 128.99; 129.41; 129.63; 132.71; 134.62; 135.58; 137.61; 158.52 (ArCH=C); 160.09 (C=O); 168.96 (C=N); IR *ν*: 1714 (C=O), 1632 (ArCH=), 1578 (C=N), 1484, 1437, 1360, 1288, 1263, 1155, 1017, 759; *R*_f = 0.62; *t*_R = 8.09; purity 99.13%; MS calcd for [M + H]⁺: C₂₀H₁₇N₂O₂ClS *m/z*: 384.88, found 385.27; anal. calcd: C 62.41, H 4.45, N 7.28, S 8.33; found: C 62.36, H 4.98, N 7.37, S 8.40%.

(**Z**)-2-(3-(2,4-Dichlorobenzyloxy)benzylidene)-6,7-dihydro-2*H*-imidazo[2,1-*b*][1,3]thiazin-3(5*H*)-one (34). Product 34 was purified by column chromatography; yellow powder, mp 160–162 °C; yield 13%; ¹H-NMR (300 MHz) δ: 2.15 (def.t, *J* = 2.43 Hz, 2H, SCH₂CH₂), 3.21 (def.t, *J* = 5.51 Hz, 2H, SCH₂), 3.60 (t, *J* = 5.64 Hz, 2H, NCH₂), 5.15 (s, 2H, CH₂O), 6.78 (s, 1H, ArCH=), 7.03 (dd, *J* = 7.71 Hz, *J* = 2.30 Hz, 1H, Ar H-4'), 7.34 (t, *J* = 8.08 Hz, 1H, Ar H-5'), 7.43 (d, *J* = 2.05 Hz, 1H, Ar H-5''), 7.47 (d, *J* = 2.31 Hz, 1H, Ar H-3''), 7.58–7.69 (m, 2H, Ar H-6'', H-6'), 7.88 (s, 1H, Ar H-2'); ¹³C-NMR (75 MHz; CDCl₃) δ: 22.92 (CH₂); 26.12 (SCH₂); 39.85 (NCH₂); 66.66 (CH₂O); 116.98; 117.13; 123.68; 125.42; 127.27; 129.22; 129.65; 129.76; 133.27; 133.33; 134.05; 135.66; 137.72; 158.26 (ArCH=C); 160.20 (C=O); 168.93 (C=N); IR *ν*: 1709 (C=O), 1634 (ArCH=), 1572 (C=N), 1486, 1443, 1294, 1258, 1161, 1045, 907, 779; *R*_f = 0.51; *t*_R = 8.81; purity 98.11%; MS calcd for [M + H]⁺: C₂₀H₁₈N₂O₂Cl₂S *m/z*: 419.3, found 419.1; CC (CH₂Cl₂ : AcOEt 1 : 1); anal. calcd: C 57.28, H 3.84, N 6.68, S 7.65; found: C 57.03, H 4.18, N 6.64, S 7.73%.

(**Z**)-2-(3-(3,4-Dichlorobenzyloxy)benzylidene)-6,7-dihydro-2*H*-imidazo[2,1-*b*][1,3]thiazin-3(5*H*)-one (35). Product 35 was purified by crystallization; yellow powder, mp 188–191 °C (acetonitrile); yield 14.94%; ¹H-NMR (300 MHz; CDCl₃) δ: 2.23–2.31 (m, 2H, SCH₂CH₂), 3.15 (def.t, *J* = 5.64 Hz, 2H, SCH₂), 3.73 (t, *J* = 5.90 Hz, 2H, NCH₂), 5.06 (s, 2H, CH₂O), 6.90 (s, 1H, ArCH=), 6.94 (dd, *J* = 7.87 Hz, *J* = 1.03 Hz, 1H, Ar H-4'), 7.26–7.32 (m, 2H, Ar H-2'', Ar H-5''), 7.45 (d, *J* = 8.20 Hz, 1H, Ar H-6''), 7.53–7.57 (m, 2H, Ar H-5', H-6'), 7.89–7.91 (m, 1H, Ar H-2'); ¹³C-NMR (75 MHz; CDCl₃) δ: 22.92 (CH₂); 26.15 (SCH₂); 39.86 (NCH₂); 68.53 (CH₂O); 116.68 (ArCH=); 117.31; 123.76; 125.51; 126.81; 129.38; 129.65; 130.53; 131.89; 132.68; 135.59; 137.26; 137.54; 158.29 (ArCH=C); 160.23 (C=O); 168.88 (C=N); IR *ν*: 1704 (C=O), 1636 (ArCH=), 1576 (C=N), 1488, 1465, 1357, 1230, 1180, 1163, 1040, 903, 795; *R*_f = 0.63; *t*_R = 8.65; purity 99.05%; MS calcd for [M + H]⁺: C₂₀H₁₆N₂O₂Cl₂S *m/z*: 419.33, found 419.24; anal. calcd: C 57.28, H 3.85, N 6.68, S 7.65; found: C 57.07, H 4.11, N 6.73, S 7.58%.

(**Z**)-4-((3-Oxo-6,7-dihydro-3*H*-imidazo[2,1-*b*][1,3]thiazin-2(5*H*)-ylidene)methyl)phenyl benzoate (36). Product 36 was purified by

column chromatography; yellow powder, mp 198–200 °C; yield 22%; ¹H-NMR (300 MHz; DMSO-*d*₆) δ: 2.18–2.22 (m, 2H, SCH₂CH₂), 3.25 (def.t, *J* = 5.50 Hz, 2H, SCH₂), 3.66 (t, *J* = 5.77 Hz, 2H, NCH₂), 6.91 (s, 1H, ArCH=), 7.39 (dd, *J* = 8.80 Hz, *J* = 1.93 Hz, 2H, Ar H-3', H-5'), 7.63 (t, *J* = 7.56 Hz, 2H, Ar H-3'', H-5''), 7.79 (t, *J* = 6.05 Hz, 1H, Ar H-4''), 8.16 (dd, *J* = 6.88 Hz, *J* = 1.65 Hz, 2H, Ar H-2'', H-6''), 8.27 (dd, *J* = 8.80 Hz, *J* = 2.48 Hz, Ar H-2', H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ: 22.86 (CH₂); 26.15 (SCH₂); 39.87 (NCH₂); 122.02; 123.07; 128.61; 129.93; 130.22; 132.04; 133.20; 133.72; 137.19; 151.97 (ArCH=C); 160.25 (C=O); 164.84 (C=O); 168.88 (C=N); IR *ν*: 1736 (COO), 1713 (C=O), 1634 (ArCH=), 1593 (C=N), 1486, 1469, 1248, 1200, 1170, 1066, 1024, 899, 712; *R*_f = 0.55; *t*_R = 7.25; purity 99.25%; MS calcd for [M + H]⁺: C₂₀H₁₆N₂O₃S *m/z*: 364.4, found 365.2; CC (CH₂Cl₂ : AcOEt 1 : 1); anal. calcd: C 65.91, H 4.43, N 7.69, S 8.80; found: C 65.61, H 4.68, N 7.45, S 8.49%.

(**Z**)-4-((3-Oxo-6,7-dihydro-3*H*-imidazo[2,1-*b*][1,3]thiazin-2(5*H*)-ylidene)methyl)phenyl 4-chloro-benzoate (37). Product 37 was purified by column chromatography; yellow powder, mp 212–214 °C; yield 27%; ¹H-NMR (300 MHz; DMSO-*d*₆) δ: 2.19–2.21 (m, 2H, SCH₂CH₂), 3.22–3.30 (m, 2H, SCH₂), 3.66 (t, *J* = 5.64 Hz, 2H, NCH₂), 6.90 (s, 1H, ArCH=), 7.39 (d, *J* = 8.80 Hz, *J* = 1.92 Hz, 2H, Ar H-3', H-5'), 7.70 (d, *J* = 8.50 Hz, *J* = 1.92 Hz, 2H, Ar H-3'', H-5''), 8.16 (d, *J* = 8.52 Hz, *J* = 1.92 Hz, 2H, Ar H-2'', H-6''), 8.26 (d, *J* = 8.80 Hz, *J* = 1.92 Hz, 2H, Ar H-2', H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ: 22.86 (CH₂); 26.15 (SCH₂); 39.86 (NCH₂); 121.91; 122.96; 127.78; 128.98; 131.58; 132.16; 133.20; 137.17; 140.26; 151.72 (ArCH=C); 160.39 (C=O); 163.98 (C=O); 168.81 (C=N); IR *ν*: 1741 (COO), 1714 (C=O), 1635 (ArCH=), 1591 (C=N), 1482, 1358, 1259, 1199, 1163, 1067, 1013, 751; *R*_f = 0.59; *t*_R = 7.99; purity 98.96%; MS calcd for [M + H]⁺: C₂₀H₁₅N₂O₃SCl *m/z*: 398.9, found 399.2; CC (CH₂Cl₂ : AcOEt 1 : 1); anal. calcd: C 60.22, H 3.79, N 7.02, S 8.04; found: C 60.08, H 4.09, N 6.93, S 8.49%.

(**Z**)-2-(3-(Benzyloxy)benzylidene)-5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]thiazepin-3(2*H*)-one (42). Product 42 was purified by column chromatography; yellow powder, mp 139–141 °C (acetonitrile, dioxane); yield 29%; ¹H-NMR (300 MHz; DMSO-*d*₆) δ: 1.78 (t, *J* = 4.74 Hz, 2H, SCH₂CH₂), 2.02 (t, *J* = 3.20 Hz, 2H, NCH₂CH₂), 3.14 (def.t, *J* = 5.38 Hz, 2H, SCH₂), 3.72 (def.t, *J* = 5.00 Hz, 2H, NCH₂), 5.11 (s, 2H, CH₂O), 6.93 (s, 1H, ArCH=), 7.07 (dd, *J* = 6.41 Hz, *J* = 2.31 Hz, 1H, Ar H-4'), 7.28–7.41 (m, 4H, Ar H-5', H-3'', H-4'', H-5''), 7.47 (dd, *J* = 9.48 Hz, *J* = 1.41 Hz, 2H, Ar H-2'', H-6''), 7.71 (d, *J* = 7.95 Hz, 1H, Ar H-2'), 7.91 (d, *J* = 1.54 Hz, 1H, Ar H-6'); ¹³C-NMR (75 MHz; CDCl₃) δ: 28.16 (CH₂); 31.03 (CH₂); 32.43 (SCH₂); 41.06 (NCH₂); 70.06 (CH₂O); 117.48; 117.82; 125.49; 127.38; 127.77; 127.85; 128.02; 128.58; 128.76; 129.67; 135.20; 136.79; 138.94; 158.84 (ArCH=C); 164.92 (C=O); 169.60 (C=N); IR *ν*: 1703 (C=O), 1632 (ArCH=), 1580 (C=N), 1488, 1437, 1337, 1287, 1262, 1178, 1154, 1022, 787, 748, 700; *R*_f = 0.84; *t*_R = 8.03; purity 98.82%; MS calcd for [M + H]⁺: C₂₁H₂₀N₂O₂S *m/z*: 364.4, found 365.3; anal. calcd: C 69.20, H 5.53, N 7.69, S 8.80; found: C 69.12, H 5.66, N 7.67, S 9.14%.

(**Z**)-2-(3-(3-Chlorobenzyloxy)benzylidene)-5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]thiazepin-3(2*H*)-one (44). Product 44 was purified by column chromatography; yellow powder, mp 129–131 °C; yield 16%; ¹H-NMR (300 MHz; DMSO-*d*₆) δ: 1.77

(def.t, 2H, SCH₂CH₂), 2.01 (def.t, 2H, NCH₂CH₂), 3.14 (def.t, *J* = 5.38 Hz, 2H, SCH₂), 3.72 (t, *J* = 4.87 Hz, 2H, NCH₂), 5.13 (s, 2H, CH₂O), 6.93 (s, 1H, ArCH=), 7.05 (dd, *J* = 7.94 Hz, *J* = 2.31 Hz, 1H, Ar H-4'), 7.32–7.45 (m, 4H, Ar H-5', Ar H-4'', H-5'', H-6''), 7.53 (s, 1H, Ar H-2''), 7.73 (d, *J* = 7.95 Hz, 1H, Ar H-6'), 7.90 (d, *J* = 1.54 Hz, 1H, Ar H-2'); ¹³C-NMR (75 MHz; CDCl₃) δ: 28.12 (CH₂); 30.98 (CH₂); 32.46 (SCH₂); 41.09 (NCH₂); 69.17 (CH₂O); 117.22; 117.91; 125.27; 125.65; 125.78; 127.63; 127.68; 128.13; 129.73; 129.85; 134.46; 135.21; 138.90; 158.52 (ArCH=C); 165.13 (C=O); 169.46 (C=N); IR *ν*: 1725 (C=O), 1649 (ArCH=), 1579 (C=N), 1488, 1372, 1235, 1161, 953, 779, 681; *R*_f = 0.83 (CHCl₃ : AcOEt 1 : 1); *t*_R = 8.58; purity 98.46%; MS calcd for [M + H]⁺: C₂₁H₁₉N₂O₂ClS *m/z*: 398.9, found 399.2; CC (CH₂Cl₂ : AcOEt 1 : 1); anal. calcd: C 63.22, H 4.80, N 7.02, S 8.04; found: C 63.45, H 5.14, N 6.86, S 7.97%.

(Z)-2-(3-(2-Chlorobenzoyloxy)benzylidene)-5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]thiazepin-3(2H)-one (45). Product 45 was purified by column chromatography; yellow powder, mp 139–141 °C; yield 23%; ¹H-NMR (300 MHz; DMSO-*d*₆) δ: 1.76 (def.t, 2H, SCH₂CH₂), 2.02 (def.t, 2H, NCH₂CH₂), 3.14 (def.t, *J* = 5.09 Hz, 2H, SCH₂), 3.72 (t, *J* = 4.61 Hz, 2H, NCH₂), 5.17 (s, 2H, CH₂O), 6.95 (s, 1H, ArCH=), 7.08 (dd, *J* = 8.20 Hz, *J* = 2.56 Hz, 1H, Ar H-4'), 7.34–7.50 (m, 3H, Ar H-5', Ar H-3'', H-5''), 7.52 (m, 1H, Ar H-4''), 7.62 (def.dd, *J* = 4.10 Hz, *J* = 1.54 Hz, Ar H-6''), 7.75 (d, *J* = 7.69 Hz, 1H, Ar H-6'), 7.94 (s, 1H, Ar H-2'); ¹³C-NMR (75 MHz; CDCl₃) δ: 28.14 (CH₂); 30.99 (CH₂); 32.43 (SCH₂); 41.08 (NCH₂); 67.25 (CH₂O); 117.49; 117.85; 125.61; 126.94; 127.69; 129.02; 129.04; 129.41; 129.76; 132.77; 134.52; 135.24; 138.87; 158.57 (ArCH=C); 165.09 (C=O); 169.52 (C=N); IR *ν*: 1725 (C=O), 1652 (ArCH=), 1576 (C=N), 1486, 1374, 1198, 1160, 1027, 967, 756, 650; *R*_f = 0.76; *t*_R = 8.56; purity 97.80%; MS calcd for [M + H]⁺: C₂₁H₁₉N₂O₂ClS *m/z*: 398.9, found 399.2; CC (CH₂Cl₂ : AcOEt 1 : 1); anal. calcd: C 63.22, H 4.80, N 7.02, S 8.04; found: C 63.54, H 5.05, N 6.77, S 8.02%.

(Z)-2-(3-(4-Fluorobenzoyloxy)benzylidene)-5,6,7,8-tetrahydroimidazo[2,1-*b*][1,3]thiazepin-3(2H)-one (47). Product 47 was purified by column chromatography; yellow powder, mp 130–133 °C (acetonitrile, dioxane); yield 13%; ¹H-NMR (300 MHz; DMSO-*d*₆) δ: 1.76 (def.t, 2H, SCH₂CH₂), 2.01 (def.t, 2H, NCH₂CH₂), 3.14 (def.t, *J* = 5.38 Hz, 2H, SCH₂), 3.72 (t, *J* = 4.87 Hz, 2H, NCH₂), 5.09 (s, 2H, CH₂O), 6.93 (s, 1H, ArCH=), 7.05 (dd, *J* = 8.20 Hz, *J* = 2.17 Hz, 1H, Ar H-4'), 7.20 (t, *J* = 8.97 Hz, *J* = 2.05 Hz, 2H, Ar H-2'', H-6''), 7.34 (t, *J* = 8.75 Hz, 1H, Ar H-5'), 7.50–7.54 (m, 2H, Ar H-3'', H-5''), 7.72 (d, *J* = 7.95 Hz, 1H, Ar H-6'), 7.91 (s, 1H, Ar H-2'); ¹³C-NMR (75 MHz; CDCl₃) δ: 28.14 (CH₂); 30.92 (CH₂); 31.01 (SCH₂); 32.44 (NCH₂); 41.07 (CH₂O); 69.36; 115.32; 115.61; 117.23; 117.94; 125.67; 127.74; 129.59; 129.69; 132.59; 135.21; 138.90; 158.64 (ArCH=C); 164.14; 164.98 (C=O); 169.55 (C=N); IR *ν*: 1705 (C=O), 1632 (ArCH=), 1570 (C=N), 1509, 1494, 1292, 1253, 1221, 1154, 1037, 781, 550; *R*_f = 0.61; *t*_R = 8.05; purity 98.01%; MS calcd for [M + H]⁺: C₂₁H₁₉N₂O₂FS *m/z*: 382.4, found 383.2; anal. calcd: C 65.93, H 5.02, N 7.32, S 8.38; found: C 65.66, H 4.89, N 7.02, S 8.11%.

Retroviral transfection. CHO K1 cells stably transfected with the human CB₁ and CB₂ receptor were generated with a retroviral transfection system as previously described.⁴⁴

Membrane preparations for CB receptor radioligand binding assays. Frozen rat brains were purchased from Pel Freez®,

Rogers, AR, USA. Rat brain cortical membrane preparations were obtained as previously described⁵² with minor modifications. Brains were thawed and then kept on ice while the cortex was dissected. The cortex was subsequently homogenized in ice-cold 0.32 M sucrose solution using an Ultra-Turrax® T25 basic homogenizer (IKA Labortechnik) for 10 s at setting 3. The suspension was centrifuged at 1000g for 5 min (4 °C), the pellet was discarded and the supernatant was subsequently centrifuged at 37 000g for 60 min (4 °C). The supernatant was discarded and the pellet was resuspended in ice-cold water, homogenized with the Ultra Turrax® and recentrifuged at 37 000g for 60 min (4 °C). The resulting membrane pellet was resuspended in Tris-HCl buffer, 50 mM, pH 7.4, and stored at –80 °C. The protein concentration was determined by the method of Lowry.⁵³ Membrane preparations of human embryonic kidney (HEK) 293 EBNA cells expressing the human CB₁, or the human CB₂ receptor, respectively, were obtained from Perkin Elmer, Boston, USA.

Radioligand binding assays at CB₁ and CB₂ receptors.

Competition binding assays were performed *versus* CB agonist radioligand [³H](–)-*cis*-3-[2-hydroxy-4-(1,1-dimethylheptyl)-phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55,940) (final concentration 0.1 nM) using rat brain cortical membrane preparations as a source for CB₁ and membrane preparations of human embryonic kidney (HEK293, 50 µg of protein per vial) cells stably expressing the human receptor subtype (50 µg of protein per vial) as a source for CB₂ receptors. Selected compounds were additionally tested at human CB₁ receptors (50 µg of protein per vial) expressed in HEK293 cells.

The assay was performed as described elsewhere.⁴⁴ Stock solutions of the test compounds were prepared in DMSO. The final DMSO concentration in the assay was 2.5%. Data were obtained from at least three independent experiments, performed in duplicate. 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 *K*_D values of 2.4 nM ([³H]CP55,940 at hCB₁) and 0.7 nM ([³H]CP55,940 at hCB₁) were used.

[³⁵S]GTPγS binding assays. Assays were performed essentially as previously described.⁵⁴ Membrane preparations of rat brain cortical membranes, or commercially available membrane preparations expressing the human CB₁ or CB₂ receptor, respectively, 5 µg per tube, were incubated with 0.1 nM [³⁵S]GTPγS (46.3 TBq per mmol, Perkin Elmer Life Sciences, Roddgau-Jügesheim, Germany) in a total volume of 200 µl of Tris-HCl buffer, 50 mM, pH 7.4, containing 1 mM EDTA, 5 mM MgCl₂, 1 mM dithiothreitol, 30 µM GDP, 100 mM NaCl, 0.5% bovine serum albumin, and test compound dissolved in DMSO. The final DMSO concentration in the assay was 1%. Nonspecific binding was determined in the presence of 30 µM of unlabeled GTPγS. Incubations were terminated after 60 min of incubation at rt by the addition of 2 ml of ice-cold buffer containing 50 mM Tris-HCl, pH 7.4, and 5 mM MgCl₂, and rapid filtration through GF/B glass fiber filter (Schleicher and Schuell, Germany) on a Brandel® 48-channel harvester, followed by two washing steps with ice-cold buffer, 2 ml each. Radioactivity on the wet filters was measured by liquid scintillation counting after punching

out the filters and incubating them for 9 h in 2.5 ml of scintillation cocktail (Ready Safe™, Beckman Coulter, USA).

cAMP accumulation assays. Cyclic AMP levels were determined in CHO cells stably expressing the human CB₁ or the human CB₂ receptor subtype, respectively, using a competition binding assay for cAMP according to the procedure described elsewhere.⁴⁴ The cAMP binding protein was obtained from bovine adrenal cortex as previously described.⁵⁵ Three separate experiments were performed in duplicate. All data were analyzed with Graph Pad Prism, Version 4.02 (GraphPad Inc., La Jolla, CA).

β-Arrestin recruitment assays. Recruitment of β-arrestin molecules to the respective receptor was detected by using β-galactosidase enzyme fragment complementation technology (β-arrestin PathHunter™ assay, DiscoverRx, Fremont, CA, USA) as previously described.^{28,40,42} Data were obtained from three independent experiments, performed in duplicate. Data were analyzed using Graph Pad Prism Version 4.02 (San Diego, CA, USA).

Abbreviations

BTEA	Benzyltriethylammonium chloride
CB	Cannabinoid
CHO cells	Chinese hamster ovary cells
GPCR	G protein-coupled receptor
GPR18	G protein-coupled receptor 18
GPR55	G protein-coupled receptor 55
HEK cells	Human embryonic kidney cells
LPI	Lysophosphatidylinositol
MAP kinase	Mitogen-activated protein kinase
NAGly	N-Arachidonoylglycine
nd	Not determined
PSB	Pharmaceutical Sciences Bonn
SARs	Structure–activity relationships
THC	Tetrahydrocannabinol

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