Cannabinoid CB$_2$ receptor ligand profiling reveals biased signalling and off-target activity

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The cannabinoid CB$_2$ receptor (CB$_2$R) represents a promising therapeutic target for various forms of tissue injury and inflammatory diseases. Although numerous compounds have been developed and widely used to target CB$_2$R, their selectivity, molecular mode of action and pharmacokinetic properties have been poorly characterized. Here we report the most extensive characterization of the molecular pharmacology of the most widely used CB$_2$R ligands to date. In a collaborative effort between multiple academic and industry laboratories, we identify marked differences in the ability of certain agonists to activate distinct signalling pathways and to cause off-target effects. We reach a consensus that HU910, HU308 and JWH133 are the recommended selective CB$_2$R agonists to study the role of CB$_2$R in biological and disease processes. We believe that our unique approach would be highly suitable for the characterization of other therapeutic targets in drug discovery research.
Target validation is an essential element of pharmacological research and drug discovery. Pharmacological intervention using chemical probes provides a powerful means to assess the temporal consequences of acute modulation of protein function under both physiological and pathological conditions. High selectivity and well-defined molecular mode of action of chemical probes are essential to translate the preclinical studies on non-human species to the patient. This type of information is, however, often lacking and reproducibility across different laboratories is sometimes difficult to obtain.

There is a great interest in the development of selective type-2 cannabinoid receptor (CB2R) agonists as potential drug candidates for various pathophysiological conditions, which include chronic and inflammatory pain, pruritus, diabetic neuropathy and nephropathy, liver cirrhosis, and protective effects after ischemic-reperfusion injury. CB2R belongs to the cannabinoid receptor family of G protein-coupled receptors, which also includes type-1 cannabinoid receptor (CB1R). Both CB2Rs are the biological target of Δ9-tetrahydrocannabinol (Δ9-THC), the main psychoactive component in cannabis. CB1R and CB2R share an overall homology of 44%, but the 7-transmembrane spanning region, which contains the ligand-binding domain, exhibits 68% similarity. CB2R is predominantly expressed on immune cells and its expression level is believed to increase in tissues upon pathological stimuli, whereas the CB1R is highly expressed in the brain. Both receptors couple to G12-13 proteins and modulate various intracellular signal transduction pathways, such as inhibition of cAMP-production, activation of ERK and G protein-coupled inward rectifying K⁺-channels (GIRKs) and recruitment of β-arrestin to the receptor, which is currently unknown which signal transduction pathways (or combinations thereof) are relevant for therapeutic purposes. In addition, some compounds may act as biased and/or protean agonists, and remarkable differences between rodent and human receptor orthologues have been noted, which are complicating the translation of results from preclinical animal models to human trials.

Different chemical classes have been described as CB2R ligands, and these are high-quality grade material, provided to each laboratory. All 18 compounds are profiled for their physico-chemical properties, absorption distribution metabolism and excretion (ADME) and pharmacokinetic parameters and cross-reactivity in the CERE panel of 64 common off-targets. Commonly used non-selective ligands, including Δ9-THC and the endocannabinoids 2-AG and anandamide (AEA, N-arachidonyl ethanolamine), CB2R agonists: SR141716A (rimonabant), and AM251; CB2R agonists: HU308, HU910, Cp127, JWH133 and AM241; and CB2R antagonists: AM630 and SR144528; see Supplementary Information, Supplementary Fig. 1 for structures. These ligands are used to explore CB2R biology and to obtain preclinical target validation of the CB2R subtypes. The high homology between the ligand binding domains of the two receptors and the overall higher tissue expression of CB2R pose challenges to develop selective ligands that target only CB2R. Yet, high selectivity is required to determine the exact role of each receptor in various (patho)physiological processes and to avoid CB2R-mediated (psychotropic) side effects caused by THC and other CB2R ligands. The need for highly selective CB2R ligands is exemplified by the scientific dispute whether the CB2R plays an important role in normal brain function or not. This whole avenue of research is currently being hampered by possible bias of using non-selective pharmacological, immunological and genetic tools and has delayed the development of novel CB2R-based drugs.

Currently, most ligands are only characterized in a binding assay and/or in a limited set of functional assays using recombinant human receptors. The results are scattered among various publications and are derived from different experimental settings, which may have led to apparent contradictory results. Conflicting results from in vivo models that employ some of the above-mentioned ligands have also been described in the literature (for a review see refs 2,24). Often, information about potential off-targets and pharmacokinetics of ligands is also lacking, which has complicated the comparison and interpretation of the data and led to confusion about which are the preferred ligands to be used for in vivo experiments aimed at validating the CB2 receptor as a therapeutic target. Unfortunately this situation, which has resulted in a loss of resources and unnecessary use of animals, is not unique to the CB2 receptor field. The US National Institutes of Health (NIH) shares these concerns from many scientists about the reproducibility issues in biomedical research and required action to counter this problem. To improve target validation and to guide the selection of the best ligand for preclinical studies, a fully detailed profile of the current ‘gold standard’ ligands is needed.

To provide important guidance for the field and to address potential species-dependent differences, we comprehensively profiled the most widely used CB2R ligands. In several independent academic and industry laboratories we investigated receptor binding of both human and mouse CB2R, as well as multiple signal transduction pathways (GTPγS, cAMP, β-AR, pERK and GIRK). Selectivity of the ligands was determined towards a customized panel of proteins associated with cannabinoid ligand pharmacology, which includes the CB2R and the major proteins of the endocannabinoid system: N-acyl ethanolamines biosynthesizing enzyme NAPE-PLD and AEA hydrolysing enzyme FAAH; 2-AG biosynthesizing enzyme DAGL and hydrolysing enzymes MAGL, ABHD6 and ABHD12, as well as towards the putative endocannabinoid transporters; AEA and 2-AG-binding transient receptor potential (TRP)-channels (TRPV1–4, TRPM8 and TRPA1) (for a review see ref. 26). In addition, off-target activity on GPR55, a receptor that binds CB2-type ligands, and on COX-2, which oxygens AEA and 2-AG, was also determined. Determination of the selectivity of CB2R ligands over these other proteins and processes involved in the endocannabinoid system, as well as over the TRP channels (which are involved in similar biological processes as the CB2Rs) is essential for the development of selective CB2R ligands and to avoid complications in the interpretation of the in vivo results obtained with these compounds.

To assess which ligands are best suited for in vivo studies, all 18 compounds are profiled for their physico-chemical properties, in vitro absorption distribution metabolism and excretion (ADME) and pharmacokinetic parameters and cross-reactivity in the CERE panel of 64 common off-targets. Commonly used non-selective ligands, including Δ9-THC and the endocannabinoids 2-AG and anandamide are also tested in vitro. All ligands are high-quality grade material, provided to each laboratory by the industry collaborator. The top three candidate CB2R agonists are further investigated at high doses in vivo to infer potential interactions with CNS CB2R. All data together results in the largest data set generated so far under the same experimental conditions for all cannabinoid receptor ligands, leading to a consensus that HU910, HU308 and JWH133 possess the best CB2R agonist profiles among the ligands tested on the basis of selectivity, balanced signalling, pharmacokinetic profile and off-target activity, and may be considered ‘gold standards’ for CB2R validation studies in mice.

Results

Physico-chemical properties. The physico-chemical properties of the 18 compounds tested are listed in Supplementary Table 1 of the Supplementary Information. Molecular weights span
a range from 312 g mol\(^{-1}\) for JWH133 up to 555 g mol\(^{-1}\) for AM251 and the polar surface area values are overall very low (8 Å for JWH133 up to 63 Å for (S)-AM1241), due to a low number of heteroatoms present in the ligands. Importantly, all CBR ligands are very lipophilic molecules, which negatively affect their solubility, ADME-properties and off-target profile. Even the lowest lipophilicity value (clogP), calculated to be 4.9 for WIN55212-2, is relatively high. The most lipophilic CBR ligand is SR144528, which exhibits an extremely high clogP value of 9.2. Consequently, only CP55940 and (rac)-AM1241 were soluble in an aqueous phosphate buffer system (pH 6.5). Despite the fact that the membrane permeation coefficient (PAMPA) \(P_{\text{eff}}\) is low for several of the molecules, most compounds are expected to be able to cross biological barriers as high percentages of the substances were found in membranes.

**Affinity and selectivity in CBR binding studies.** To determine the affinity and selectivity of the 18 substances, we performed \(^{3}\text{H}\)-CP55940 displacement assays using membrane fractions of CHO cells expressing recombinant human CBRs and CBRs, in two independent laboratories. In addition, mouse brain and spleen were used as source of mouse CBR and CBR, respectively.

Using the Pearson correlation analysis, we found a statistically significant correlation between the binding affinities between the different labs (Pearson coefficient: 0.9304 (hCB2R), 0.6648 (hCB1R) and 0.7720 (mCB1R), see Supplementary Fig. 2). Figure 1 depicts the selectivity of the ligands for the CBR versus CBR. We found that HU210 > CP55940, WIN55212-2 > Δ⁹-THC were the highest affinity non-selective human CBR ligands. Conversely, HU308, HU910 and JWH133 were the most selective human CBR ligands (Supplementary Table 2), possessing 278-, 166- and 153-fold higher respective affinities for CBR than for CBR. Notably, JWH015 and Gp-1a were less than 30-fold selective for CBR.

Importantly, the binding selectivity of the ligands for mouse CBR over mouse CBR appeared to be greatly reduced for all ligands (> 100 fold), except AM630 and SR144528, which are actually more selective on mCB2R than on hCB2R. The most selective agonists on mCB2R were (rac)-AM1241 (66-fold), JWH133 (40-fold) and Gp-1a (20-fold). As expected, AEA and 2-AG, the endogenous ligands of CBR and CBR, were non-selective and showed moderate binding affinities towards both receptors (pKi ~ 7).

**Activity and selectivity of CBR signalling pathways.** To determine the functional activity and selectivity (towards CBR over CBR) of the ligands we performed five different assays (GTP\(\gamma\)S, cAMP, β-AR, pERK and GIRK) on both human CBR and human CBR (Supplementary Tables 3–7). All ligands were tested on CAMP signalling on both mouse CBRs and HU910, HU308 and JWH133 were tested on G-protein activation and β-arrestin recruitment on mCB2R, to determine interspecies behaviour of the ligands. Efficacy of the ligands is normalized to the effect produced by CP55940 (10 μM) in all assays; however, it should be noted that efficacy is relative by definition, and is dependent on the reference ligand used as well as the assay conditions.

For both human and mouse CBR, the potency of the ligands correlated with their binding affinity in most assays, except for β-AR and GIRK signalling (Supplementary Fig. 3). Graphs showing the pEC50 values of the reference ligands for all assays are shown in Fig. 2a–d.

CP55940 and HU308 behaved as potent full agonists at hCB2R in the GTP\(\gamma\)S assay (Supplementary Table 3), while WIN55212-2 displayed as a partial agonist. HU910 behaved as a partial CBR agonist as well, but was, together with HU308 and JWH133, the most selective for CBR in this assay (185- and 193-fold, respectively). Of note, JWH133 was considered functionally inactive on hCB2R, because its maximal effect was only 20% at 10 μM. On mCB2R, both HU308 and JWH133 were full agonists, but HU910 remained partially active. The potency of all three ligands was similar for human and mouse receptors.

In contrast to previous reports, Gp-1a acted as an inverse agonist on CBR, but was inactive at CBR. Both THC and the endocannabinoids AEA and 2-AG acted as partial agonists on both receptors with similar potency.

In the cAMP assay (Supplementary Table 4) all CBR agonists displayed higher selectivity (> 1,000-fold) and higher efficacy, than in the GTP\(\gamma\)S assay, reflecting substantial signal amplification in this pathway. Only (rac)-AM1241 remained a partial agonist in the cAMP assay. Upon comparison of the efficacy of the ligands between species, in general it appears that many ligands on the mouse CBRs are partial agonists on cAMP, in contrast to the human CBRs. This difference in efficacy might be a result of a difference in CBR expression levels. Differences in expression levels may also account for the interspecies differences displayed by (rac)-AM1241, which was previously reported as a prototypic agonist (a prototypic agonist shows differences in signalling due to differences in experimental conditions, whereas a true biased agonist has signalling preference due to conformational changes of the receptor). HU910 was found to bind with similar affinity to both mouse CBRs, but was inactive on mCB2R in the cAMP assay. HU308, JWH015 and (rac)-AM1241 were the most selective agonists for hCB2R in this assay, whereas CP55940, WIN55212-2 and HU210 displayed the highest potency. HU910, HU308 and JWH133 were the most selective on the mCB2R. Of note, AEA and 2-AG were relatively weak partial agonists, especially on human and mouse CBR (pEC50 < 5.2 and Emax < 70%).

All ligands modulated β-AR recruitment (Supplementary Table 5) to the membrane in CHO cells expressing human CBR or CBR. CP55940 was the most potent ligand in this assay, followed by WIN55212-2. CP55940 acted as full agonist at both receptors, but WIN55212-2 displayed partial agonism at
CB2R agonists, whereas CP55940 lacked selectivity in this assay. 

WIN55212-2 and CP55940 activated the GIRK channels with the highest potency, but as expected, both were highly potent and efficacious at CB1R as well.

1JWH133 nor THC activated the GIRK pathway at all. JWH015 was the most selective agonist in this assay, followed by HU308. Of note, JWH133, HU308 and HU910 were all significantly less potent on mCB2R in β-AR recruitment.

WIN55212-2 was a full agonist in the pERK assay (Supplementary Table 6) and demonstrated 86-fold selectivity for the human CB2R, whereas CP55940 lacked selectivity in this assay. HU308 and JWH133 were potent and selective CB2R full agonists, whereas Δ9-THC and (Rac)-AM1241 acted as partial agonists on the pERK signalling cascade. Interestingly, HU910, AEA and 2-AG had low potency in this assay (pEC50 < 5.5), but HU910 and 2-AG acted as full agonists at high concentrations.

Most ligands appeared to be less potent and less CB2R-selective in the GIRK assay (Supplementary Table 7). For example, neither JWH133 nor THC activated the GIRK pathway at all. JWH015 was the most selective agonist in this assay, followed by HU308. WIN55212-2 and CP55940 activated the GIRK channels with the highest potency, but as expected, both were highly potent and efficacious at CB2R as well.

The high variability in potency and efficacy that the CB2R agonists displayed across the different signalling pathways strongly suggests biased signalling. To quantify this ligand bias towards distinct signal transduction pathways, we performed operational analysis based on van der Westhuizen et al. (Supplementary Tables 8–11) (ref. 30). This analysis is based on the operational model of Black and Leff31, which calculates signal transduction strength on a given pathway, taking into account (a) the maximal effect of the system used, (b) the agonist concentration, (c) the agonist’s maximum efficacy, (d) the ligand affinity for the receptor and (e) the transducer slope.

In order to eliminate system and observation bias, such as the level of amplification between signalling pathways or assay sensitivity, we used CP55940 as a reference compound, because it was the only compound that behaved as a full agonist with comparable potency in all assays, except for the cAMP assay. The ΔΔlogR values resulting from this operational analysis are graphically shown in Fig. 3. The operational analysis on hCB2R revealed that THC showed statistically significant bias towards pERK signalling compared to β-arrestin and GTPγS. In addition, THC did not activate GIRK, indicative of high bias against this pathway. (rac)-AM1241 was biased towards β-arrestin coupling and pERK signalling compared to GIRK channel activation. JWH133 was moderately biased towards β-arrestin compared to GIRK, whereas both WIN55212-2 and JWH015 showed preference for GIRK compared to cAMP signalling. AEA showed preference for pERK and GIRK signalling compared to cAMP, whereas 2-AG was significantly biased towards GIRK compared to G-protein signalling. Upon comparison between β-AR coupling and cAMP signalling, all ligands appear to be significantly biased. This observation is, however, confounded by the fact that CP55940, which is used as the reference ligand, has an exceptionally high potency in the cAMP assay compared to the other signal transduction pathways, and might be in fact biased itself towards the cAMP pathway.
pathway. Of note, HU910 and HU308 were well-balanced ligands without significant bias towards any signal transduction pathway on hCB2-R. Of note, HU910, HU308 and JWH113 were significantly biased towards G-protein signalling over β-AR coupling and cAMP signalling on the mCB2-R highlighting a potentially important species difference.

Off-target activity in the endocannabinoid system. To rule out any indirect effects of the ligands on CB2-R and CB1-R, we investigated off-target activity on endocannabinoid-regulating enzymes, as well as their effects on AEA reuptake inhibition. None of the ligands showed any off-target activity on a panel of serine hydrolases, determined in a competitive activity-based protein profiling (ABBP) assay in mouse brain proteome up to a concentration of 10 μM (Supplementary Fig. 4A). In addition, none of the ligands showed any significant effect at a concentration of 10 μM when tested on NAPE-PLD, DAGL and MAGL-overexpressing cells (Supplementary Table 12). The compounds were also tested on FAAH activity using U937 cell homogenate at a concentration of 5 μM. Only AM251 and Gp-1a showed partial inhibition of FAAH activity (~30–40%, Supplementary Fig. 4F).

In addition, none of the agonists had significant activity on ABHD6-, ABHD12- and COX2 activity up to a concentration of 5 μM for COX2 and 10 μM for the ABHDs (Supplementary Fig. 4B–E). In contrast, the antagonists SR141716A, AM251 and Gp-1a, which are the structurally most similar ligands in this panel of ligands, inhibited ABHD12 in the micromolar range with IC50 values of 6.1, 1.6 and 0.8 μM, respectively (Supplementary Table 12). AM251 was the only compound of the ligands tested that had high efficacy on GPR55 in β-arrestin recruitment (82 ± 9%), albeit with low potency (pEC50 = 5.49 ± 0.09, see Supplementary Table 13).

AEA reuptake inhibition was determined in three different human cell lines: monocyte-like U937 cells, mast cell-like HMC-1 cells and keratinocyte-like HaCaT cells. In U937 and HaCaT cells, some of the ligands possessed micromolar potency, including AM251, SR141716A, Gp-1a, HU308 and HU910 (Supplementary Table 14). In HMC-1 cells, which lack FAAH expression32, all tested ligands, except SR141716A, were weakly active or inactive at a concentration of 5 μM, which indicates a potential role of FAAH in the inhibition of AEA uptake in U937 and HaCaT cells. In agreement with this, the most active AEA reuptake inhibitors AM251 and Gp-1a partially inhibited FAAH at 5 μM in U937 cell homogenate (Supplementary Fig. 4F). Of note, SR144528 was inactive up to a concentration of 10 μM in all cell lines, whereas SR141716A showed FAAH-independent micromolar effects on AEA reuptake in all cells.

TRP-channels. As AEA and 2-AG activate some TRP channels, these channels may be regarded as ionotropic cannabinoid receptors. Here, we tested our ligands on six different TRP channels (TRPV1–4, TRPA1 and TRPM8). We found that most TRP channels were activated by one or more ligands, apart from TRPA1 that was activated by all of them (Supplementary Table 15). HU308 was the most selective agonist that activated TRPM8, which was activated by all them (Supplementary Fig. 4F).

Off-target panel (CEREP panel). The CEREP panel served to screen off-target activity on 64 proteins, which are associated with
common adverse side effects in humans. JWH133 was identified as the most selective ligand with no off-targets detected in this panel. The summary of all off-targets is shown in Supplementary Table 16, in Supplementary Table 20 all CEREP data are shown. In contrast, CP55940 was the most non-selective ligand of which we detected 17 off-targets with more than 50% inhibition at 10 μM. HU910 and HU308 hit nine and four off-targets in this panel, respectively. However, of the nine off-targets of HU910 in this panel only the dopamine uptake reporter displayed an IC\textsubscript{50} of <10 μM (IC\textsubscript{50} = 1.40 μM). Therefore, these off-targets are not likely to be physiologically relevant at 10 μM. The CB\textsubscript{2}R-selective antagonist SR144528 had only two off-targets. Of note, the adenosine A\textsubscript{3} receptor was the most common off-target (Supplementary Table 16). The physiological relevance of this observation is currently unclear, although it has previously been published that the endocannabinoid 2-AG has allosteric activity on this receptor\textsuperscript{34}.

**In vitro** DMPK and pharmacokinetics. The high overall lipophilicity (see above) may strongly influence other ADME properties. Metabolic stability in human and mouse microsomes and hepatocytes is low for many of the ligands as indicated by their high *in vitro* clearances (Supplementary Table 17). Some compounds, such as AEA, suffered from high chemical instability even in dimethylsulfoxide (DMSO) stock solution. For most ligands, except Gp-1a, microsomal clearances seem to over-predict the corresponding values in both human and mouse hepatocytes. The fraction unbound (Fu) was either very low or not measured for many compounds due to their very high lipophilicity. None of the molecules is a strong human or mouse P-gp substrate. In combination with their low polar surface areas, the ligands are, therefore, likely to reach the brain.

We determined the primary pharmacokinetic parameters of the compounds (Supplementary Table 18). A mixture containing 15% DMSO and 85% PEG400 was used as a vehicle to dissolve the ligands at 1–2 mg kg\textsuperscript{-1} for intravenous (i.v.) administration. *In vivo* clearances in mice were very high and span a range from the lowest value of 0.17 h\textsuperscript{-1} kg\textsuperscript{-1} for HU910 up to 6.91 h\textsuperscript{-1} kg\textsuperscript{-1} for (S)-AM1241. The volume of distribution was high (>31 kg\textsuperscript{-1}) for (S)-AM1241, AM630, Gp-1a and JWH015, moderate (1–31 kg\textsuperscript{-1}) for AM251 and JWH133 and low (<11 kg\textsuperscript{-1}) for HU910 and SR144528. This resulted in the longest half-life for HU910 (7 h), whereas JWH133 had a half-life of only 1 h. As reasonable C0 values, extrapolated from Cmax values, can be reached for all compounds (see Supplementary Table 18), the rather short *in vivo* half-lives raise the possibility that published *in vivo* efficacy data using these molecules might be rather C0 than AUC driven. Future experiments using accurate concentration/effects relationships might answer this question.

Following oral administration using aqueous microsuspensions in rodents, absorption was strongly influenced by the physicochemical properties of the compounds, formulation and feed conditions of the animals (Supplementary Table 19). HU910 was suspended in ethanol/cremophor EL/0.9% NaCl (5/5/90% w/w) whereas HU308 and JWH133 were suspended in an aqueous gelatine/NaCl vehicle (7.5/0.62% w/w). Maximal plasma concentration peaked ~1 h after administration with Cmax ranging from 201 to 2070 ng ml\textsuperscript{-1}. Half-lives were comparable to i.v. administration. Bioavailability was not calculated as these were separate experiments. Taken together these data suggest a wide variety of application format for *in vivo* experiments if care is taken on the formulation aspects. Determination of the plasma concentrations and pharmacokinetic behaviour seems however warranted.

***In vivo* selectivity of HU308 HU910 and JWH133.** Finally, to determine whether the three most selective agonists, HU308, HU910 and JWH133, elicited cannabinimimetic pharmacological effects *in vivo*, these compounds were tested in assays highly sensitive to CB\textsubscript{2}R activity (catalepsy, antinociception and hypothermia). HU210, a non-selective, highly potent CB\textsubscript{2}R/CB\textsubscript{1}R agonist was used for comparison. In addition, as binding data had suggested affinity for mCB\textsubscript{1}R (K\textsubscript{D} = 6.14 ± 0.13), HU910 was tested for antagonistic effects in these *in vivo* measures, and compared with SR141716A.

As shown in Fig. 4, HU210 dose-dependently elicited catalepsy (Fig. 4a; F (7,42) = 60.7, P < 0.0001 (Dunnett’s test); ED\textsubscript{50} (95% CL) = 0.19 (0.14–0.27) mg kg\textsuperscript{-1}, antinociception (Fig. 4b; F (7,35) = 257.5, P < 0.0001 (Dunnett’s test); ED\textsubscript{50} (95% CL) = 0.41 (0.31–0.54) mg kg\textsuperscript{-1}) and hypothermia (Fig. 4c; F (7,35) = 97.7; P < 0.0001 (Dunnett’s test); ED\textsubscript{50} (95% CL) = 0.35 (0.30–0.41) mg kg\textsuperscript{-1}). In contrast, HU308, HU910 and JWH133 did not produce detectable catalepsy (Fig. 4a), antinociception (Fig. 4b) or hypothermia (Fig. 4c) within the dose range tested (1–100 mg kg\textsuperscript{-1}).

To test whether HU910 behaves as a CB\textsubscript{1} receptor antagonist *in vivo*, we tested whether 30 mg kg\textsuperscript{-1} HU910 would antagonize the pharmacological effects of 1.7 mg kg\textsuperscript{-1} HU210 (that is, an approximate ED\textsubscript{50} dose). Whereas SR141716A (3 mg kg\textsuperscript{-1}) significantly antagonized the cataleptic (Fig. 4d; F (1,10) = 46.7, P < 0.0001 (Holm-Sidak’s test), antinociceptive (Fig. 4e; F(1,10) = 39.7, P < 0.0001 (Holm-Sidak’s test)) and hypothermic (Fig. 4f; F (1,10) = 116.1; P < 0.01 (Holm-Sidak’s test)) effects of HU210, HU910 did not significantly reduce the magnitude of HU210-induced catalepsy (Fig. 4g; P = 0.12 (Holm-Sidak’s test)), antinociception (Fig. 4h; P = 0.19 (Holm-Sidak’s test)) or hypothermia (Fig. 4i; P = 0.40 (Holm-Sidak’s test)). These results indicate that HU308, HU910 and JWH133 lack CB\textsubscript{2}R activity at relevant concentrations *in vivo*.

**Discussion**

Drug discovery research has focused on the design and synthesis of selective cannabinoid CB\textsubscript{2}R agonists. Selective activation of this receptor has been associated with anti-inflammatory and tissue protective effects without inducing CB\textsubscript{2}R-mediated psychoactive side effects. This concept has been supported by the use of CB\textsubscript{2}R knock-out mice showing enhanced pathology in disease models, such as heart, liver or kidney injury and inflammation. It is unclear why two different CB\textsubscript{2}R agonists lacked efficacy in phase 2 clinical pain trials\textsuperscript{35,36}, despite compelling proof-of-concept data obtained in preclinical studies. This lack of translation not only suggests possible deficiencies in the predicative utility of the preclinical models, but also that improved understanding of the molecular actions of CB\textsubscript{2}R agonists is needed. In addition, selective CB\textsubscript{2}R ligands are essential to determine whether CB\textsubscript{2}R has a physiologically relevant role in the normal brain function, which is currently under intense scientific debate\textsuperscript{22}. To answer this question, a truly CB\textsubscript{2}R-selective ligand is needed to avoid confusion caused by the use of non-selective cannabinoid ligands\textsuperscript{23}.

Here, we have comprehensively characterized a set of 18 CBR ligands for their physicochemical properties, *in vitro* molecular pharmacology, off-target profile and pharmacokinetics to guide the selection of the optimal ligands to perform preclinical proof-of-concept studies. An important finding of our study is that most agonists display reduced selectivity in binding affinity and functional efficacy on the mouse CB\textsubscript{2}R versus CB\textsubscript{1}R compared to the human orthologues while the antagonists display opposite behaviour. This observation may potentially be explained by the fact that the agonists stabilize different
receptor conformations than antagonists (inverse agonists) by interacting with different (species specific) amino acids in the binding pocket.

The reduced selectivity of the agonists for the mCB2R is a limitation that needs to be taken into account, especially when designing studies to investigate (neuro)inflammation in mice. In contrast to previous reports that classify Gp-1a as a CB2R agonist, we found Gp-1a to be a functional CB2R and CB1R antagonist (inverse agonist) in all functional assays both on human and mouse orthologues.

Another important finding in the present study is the provocative evidence indicating biased signalling of CB2R agonists. For target validation it would be advisable to use a balanced ligand, instead of a strongly biased agonist, until it is clear that activation of a specific pathway is desired, because this may complicate the translation to the human situation. In our ligand set, THC, 2-AG and (rac)-AM1241 behaved as the most biased agonists on hCB2R, with each stimulating their most preferred pathway > 100-fold stronger than their least preferred pathway. Of note, 2-AG and AEA had distinct profiles in signalling pathway activation, which might open possibilities to explain ligand diversification of CBRS. Importantly, we found that HU308 and HU910 showed differences in signalling preference between the human and mouse CB2R, being well-balanced agonists in all five signal transduction pathways on hCB2R, but significantly biased on mCB2R towards G-protein activation compared to β-arrestin recruitment and cAMP signalling. The consequences of these interspecies differences in signalling preference for the translation of preclinical models to the clinic needs to be taken into account when testing novel drug candidates.

In addition, we found that SR144528 is a very effective antagonist of CB2R-mediated modulation of cAMP signalling, but less so on other signal transduction pathways of the same receptor (GIRK and pERK). This signalling-specific inhibition should also be taken into account when studying CB2R agonists that preferably act through these mechanisms.

To determine off-target activity of the cannabinoid reference library, we tested them on a customized panel of proteins that are associated with cannabinoid ligand pharmacology, including GPR55, proteins involved in the biosynthesis and metabolism of endocannabinoids (DAGL-α, NAPE-PLD, MAGL, ABHD6, ABHD12, COX-2, FAAH and endocannabinoid transporter activity) and the TRP ion channel family. In combination with the additional off-target data we collected using the CEREP panel, we found that most ligands displayed a rich polypharmacology (see for a summary of all off-targets per compound Fig. 5 and Supplementary Table 13). Remarkably, the highly

![Figure 4](image-url) **Figure 4 | Comparison of in vivo cannabimimetic pharmacological effects.** HU210 dose-dependently elicited catalepsy (a), antinociception (b) and hypothermia (c), but HU308, HU910 and JWH133 lacked appreciable pharmacological effects in each assay. Whereas pretreatment with SR141716A (3 mg kg⁻¹) attenuated the magnitude of cataleptic (d), antinociceptive (e) and hypothermic (f) effects of HU210 (1.7 mg kg⁻¹), HU910 did not significantly reduce HU210-induced catalepsy (g), antinociception (h) or hypothermia (i). Filled symbols denote significance versus the respective vehicle injection for each drug (a–c). ***P<0.0001 HU210 versus respective pretreatment (PTx) (Holm-Sidak’s test). ****P<0.0001 versus vehicle/HU210 (Holm-Sidak’s test). Sample sizes = 8 mice/group (a–c) and 6 mice/group (d–i). All values reflect mean ± s.e.m.
potent, widely used CB1 agonist CP55940 was the least selective compound at 10 μM. Consequently, it would be advisable to use it only at low concentrations (for example, <100 nM) in in vitro and in vivo experiments. Furthermore, the fact that the potent CB2R antagonists AM251 and SR141716A display CB2R antagonism at low micromolar concentrations should be taken into account in the experimental design of in vitro-, and possibly in vivo experiments. Additional off-targets of CB2R antagonists may further complicate the interpretation of results. For example, both antagonists, together with Gp-1α, target ABHD12, an enzyme that hydrolyses 2-AG (Ref. 38), and may therefore increase endogenous 2-AG levels in a cell-selective manner. The low nanomolar effects of SR141716A on TRPM8 activation should also be kept in mind using cell lines expressing functional TRPM8. However, this off-target may not be functionally relevant in vivo because no significant effects of SR141716A on normal temperature regulation have been reported in rodent or human studies. Finally, AM251 demonstrated also some agonistic activity on GPR55 at high concentrations.

The CB2R agonists investigated in the present study displayed diverse physico-chemical properties and pharmacokinetics (p.o. or i.p. T\textsubscript{i}, and oral bioavailability), which were far from optimal and deviate from standard criteria of druglikeness\textsuperscript{39}. These factors should be taken into account during the design of in vivo studies. Nevertheless, our PK experiments have proven that effective drug concentrations can be achieved after intravenous as well as oral administration. Likely this can also be achieved using alternative application routes such as food admix, i.p. and s.c. However, formulations might need to be optimized for each compound and plasma concentrations need to be monitored during the course of the in vivo studies. The more prolonged sustained beneficial effect of HU910 in liver post-optimized for each compound and plasma concentrations need to be mixed, i.p. and s.c. However, formulations might need to be intravenous as well as oral administration. Likely this can also be achieved using alternative application routes such as food admix, i.p. and s.c. However, formulations might need to be optimized for each compound and plasma concentrations need to be monitored during the course of the in vivo studies. The more prolonged sustained beneficial effect of HU910 in liver post-optimal flushing path, on hCB2R, (c) minimal number of off-target activities at their active concentrations, (d) reasonable pharmacokinetics and (e) lack of functional in vivo pharmacological effects indicative of CB2R activity. Of the antagonists tested, SR144528 is the most suitable for use because of its high selectivity profile for CB2R in both humans and mice.

On a final note, the collaborative effort between multiple independent academic laboratories and industries to reach consensus via multicentric profiling on the key properties of widely used CBR ligands in the ever-growing field of (endo)cannabinoid research provides substantial knowledge on CB2 receptor pharmacology, which may improve data reproducibility in the field. Moreover, our unique approach may serve as a useful strategy to investigate other classes of molecules with therapeutic relevance. Such an effort is deemed necessary to allow a successful transfer of preclinical data to the patient’s bedside.

### Methods

#### General materials.

\[ ^{3}H \] CP55940 (specific activity 141.2 Ci mmol\textsuperscript{-1}) and GF-B/GF-C filters were purchased from Perkin Elmer (Waltham, MA, USA). Anandamide [arachidonyle-5,6,8,9,11,12,14,15-\textsuperscript{3}H] AEA (specific activity 200 Ci mmol\textsuperscript{-1}) was purchased from ARC (St Louis, MO, USA). Bromochromic acid (BCA) and BCA protein assay reagent were obtained from Pierce Chemical Company (Rockford, IL, USA). The PathHunter CHO-K1 CNR1, CNR2 and mCNR2 β-Arestin Cell Line (catalogue numbers 93-0959C2, 93-0706C2 and 93-0472C2, respectively) and the PathHunter detection kit (catalogue number 93-0001) were obtained from DiscoveRx. Cell culture plates were purchased from Sarstedt (catalogue number 83.3902) and 384-well white walled assay plates from Perkin Elmer (catalogue number 6007680). Cannabinoid reference ligand CP55940, (Rac)-AM1241, AEA and 2-arachidonoylglycerol (2-AG) were obtained from Sigma Aldrich (St Louis, MO, USA), HU9113 and Gp-1α from Tocris Bioscience (Bristol, UK), and SR14128, (S)-AM1241 from Cayman Chemical Company (Ann Arbor, MI, USA) and HU308, HU910, HU210, SR141716A, AM630, WIN55212-2 mesylate, and AM251 were obtained from Hoffman-La Roche (Basel, Switzerland). A\textsuperscript{2}-THC and JWH015 were synthesized according to published procedures (see below)\textsuperscript{40–42}. Not all compounds were tested in all labs due to legal restrictions. URB597 and J2L184 were from Cayman Chemicals (Ann Arbor, MI, USA). Activity-based probes for serine hydrolases MB064 and TAMRA-FP were synthesized according to literature\textsuperscript{43}, or were bought from Thermo Fischer Scientific (Waltham, MA, USA), respectively. All buffers and solutions were prepared using MilliQ water (deionized using a MilliQ A10 Biocel, with a 0.22 μm filter) and analytical grade reagents and solvents. Buffers are prepared at room temperature (RT) and stored at 4°C, unless stated otherwise.

#### Synthetic procedures general remarks.

All reactions were performed using oven or flame-dried glassware and dry solvents. Reagents were purchased from Sigma Aldrich, Acros and Merck and used without further purification unless noted otherwise. All moisture sensitive reactions were performed under an argon atmosphere. Traces of water were removed from starting compounds by co-evaporation with toluene. \( ^{1}H \) and \( ^{13}C \) NMR spectra were recorded on a Bruker Av 400 MHz spectrometer at 400.2 \( ^{1}H \) and 100.6 \( ^{13}C \) MHz. Chemical shift values are reported in ppm with tetramethylsilane or solvent resonance as the internal standard (CDCl\textsubscript{3}, \( \delta \) 7.26 for \( ^{1}H \), \( \delta \) 77.0 for \( ^{13}C \). CDOD-: \( \delta \) 3.31 for \( ^{1}H \). Data are reported as follows: chemical shifts (\( \delta \), multiplicity (s = singlet, d = doublet, dd = double doublet, td = triple doublet, t = triplet, q = quartet, quint = quintet, br = broad, m = multiplet), coupling constants (Hz), and integration. HPLC purification was performed on a preparative LC-MS system (LichroCart 1200Series with a LichroCART 1204-4.6 Quadruple MS detector). High resolution mass spectra were recorded on a Thermo Scientific LTQ Orbitrap XL. Flash chromatography was performed using SiliCycle silica gel type SiliaFlash.
Cell culture and membrane preparation. CHOK1hCB1_bgal, CHOK1hCB2_bgal and CHOK1mCB1_bgal cells (source: DiscoveRx, Fremont, CA, USA) were cultured in Ham’s F12 Nutrient Mixture supplemented with 10% fetal calf serum, 1 mM glutamine, 50 μg ml⁻¹ penicillin, 50 μg ml⁻¹ streptomycin, 300 mg ml⁻¹ hygromycin and 800 μg ml⁻¹ geneticin in a humidified atmosphere at 37 °C and 5% CO₂. Cells were harvested twice a week by trypsinization twice of 1:20 on 10-cm plates by trypsinization. For membrane preparation the cells were subcultured 1:10 and transferred to large 15-cm diameter plates. For membrane preparation, the cells were detached by scraping them into 5 ml phosphate-buffered saline (PBS), collected and centrifuged at 1000 g for 5 min. Pellets derived from 30 plates were added together and resuspended in 20 ml cold buffer (50 mM Tris- HCl, 5 mM MgCl₂, pH 7.4). An UltraTurrax homogenizer was used to homogenize the cell suspension. Membranes and the cytosolic fraction were separated by ultracentrifugation (31,000 rpm, with a Ti-70 rotor in a Beckman Coulter Ultracentrifuge) at 4 °C for 20 min. The supernatant was discarded and the pellet was resuspended in 10 ml of the same buffer and the homogenization and centrifugation steps were repeated. Supernatant was discarded and the pellet was resuspended in 5 ml buffer. Aliquots of 200 μl were frozen at −80 °C until further use. Protein concentration was determined using the BCA method.44

The basal level of GTP binding was measured by an [35S]GTP γS assay. G-protein activation by the receptor is measured by the binding of radiolabelled GTP, [35S]GTP γS, to the receptor.45 Five micrograms of homogenized CHOK1hCB1_bgal membranes in 20 μl assay buffer (50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, 150 mM NaCl, 1 mM EDTA, 0.05% BSA and 1 mM DTT, freshly prepared every day) were pretreated with 5 μg saponin and 1 mM GTP. To determine the EC₅₀ and Emass values of cannabinoid reference ligands, the samples were exposed to 10 μM of each antagonist (single point assay) or in increasing concentrations of each agonist and incubated for 30 min in a humidified atmosphere at 37 °C and 5% CO₂. In the antagonistic assays, the cells were exposed to 10 μM of each antagonist (single point assay) or in increasing concentrations of each agonist (agonistic assay) and incubated for 30 min in a humidified atmosphere at 37 °C and 5% CO₂. In the agonistic assays, the basal level of GTP binding was measured in untreated membrane protein. AEC80 concentration of CP55940 (25 nM for CHOK1hCB1_bgal and 46 nM for CHOK1hCB2_bgal). The cells were incubated for 90 min in a humidified atmosphere at 37 °C and 5% CO₂. Compounds in DMSO stocksolutions were added using a HP D300 Digital Dispenser (Tecan, Männedorf, Switzerland).

Endocannabinoids 2-AG and anandamide (stocksolutions in acetone/tritile) were added manually. The final concentration of organic solvent per assay point was <0.1%. β-Galactosidase enzyme activity was determined by using the PathHunter Detection mixture, according to the kit's protocol.46 Detection mixture, 12 μl per well, was added and the plate was incubated for 1 h in the dark at room temperature. Chemiluminescence, indicated as relative light unit, was measured on an EnVision multilabel plate reader (Perkin Elmer, MA, USA).
The time-resolved energy transfer was measured using an LF502 Nanoscan Human cAMP assay. Ethical approval was given by the Ministry of Health, n. 47/2014/PR (deadline 17 November 2019). Neurodegenerativi in modelli animali di Alzheime’ by Mauro Maccarrone. Via Indipendenza 11 Calco (Lecco) 23885 Italy), at the Santa Lucia Foundation counter (Perkin-Elmer Life Sciences, Boston, MA, USA). Brain and spleen tissue concentration of CP55–940 (1 nM CP55940) was used in order to achieve a better signal-to-background ratio, due to the lower rate of AEA uptake. The reaction was stopped by rapid filtration over UniFilter-96 GF/C filters (Perkin Elmer) pre-soaked with PBS 1% BSA. Cells on filters were washed three times with 100 µl ice-cold PBS buffer containing 1% fatty acid free BSA. After drying, 5 µl MicroScint 20 scintillation cocktail (Perkin Elmer, Waltham, MA, USA) was added to the wells and the plate was sealed. Radioactivity was measured by liquid scintillation counting on a Perkin Elmer Wallac Trilux MicroBeta 1450 during 2 min. Samples were measured in triplicates in n = 3 independent experiments except the screening run (n = 1). Each run was validated using the positive controls OMDM-2 and UCM707 at a concentration of 10 µM reaching 59.7 ± 6.6% (n = 7) and 71.5 ± 0.8% (n = 7) respectively.

**NAPE-PLD inhibition.** Full length human cDNA NAPE-PLD was obtained from Natsuo Ueda26 and cloned into mammalian expression vector pcDNA3.1, together with the Flag- and the C-terminal Flag-tagging sequence. All plasmids were grown in XL-10 Z-competent cells and prepped (Maxi Prep, Qiagen). Sequence analysis for the confirmation of the sequences was performed at the Leiden Genome Technology Centre. HEK293T cells (source: Dutch Cancer Institute) were cultured at 37 °C and 5% CO2 in DMEM with glutamin, penicillin (100 units/ml), streptomycin (100 µg/ml) and 10% New Born Calf Serum iron supplemented (HyClone SHC30072.03). Cells were passages twice a week to achieve appropriate confluence. Twenty-four hours before transfection 10⁶ cells were seeded on a 15 cm dish. Two hours before transfection, the medium was refreshed. Transfection is performed with PEI in a ratio of 3:1 with human NAPE-PLD or Mock pcDNA3.1Neo. 20 µg per dish. Medium is refreshed after 24 h and cells are harvested after 72 h. Cell suspensions are centrifuged at 1,000g for 10 min, supernatant removed and pellets frozen at −80 °C until further use.

Cell pellets are re-suspended in lysis buffer 1: 20 mM Hepes, 2 mM DTT, 0.25 M sucrose, 1 mM MgCl₂, 2.5 µM β-mercaptoethanol and incubated 30 min on ice. The cytosolic fraction (supernatant) is separated from the membranes by ultracentrifugation (32,000 rpm for 30 min 100,000g). The pellet is resuspended in buffer 2: 20 mM Hepes, 2 mM DTT (membrane fraction). All samples are stored at −80 °C. Enzyme concentrations are determined using a Bradford assay.

The membrane protein fraction from transient overexpression of NAPE-PLD in HEK293T cells was diluted to 0.4 mg/ml–1 in assay buffer: 50 mM Tris-HCl (pH 7.5), 0.02% Triton X-100, 150 mM NaCl (ref. 60). The substrate PED6 (Invitrogen) 10 mM stock was consecutively diluted in DMSO (25 x) and in assay buffer (10 x). Relevant concentrations of compounds are prepared in DMSO. This series is performed in 30 µl final volume, containing 400 µg membrane protein. The compound or DMSO is incubated with membrane protein lysate (final concentration 0.04 mg/ml–1) for 30 min at 37 °C. A sample without membrane protein lysate is incorporated for background subtraction. Then, substrate is added (final concentration 1 µM) and the measurement is started immediately on a TECAN infinite M1000 pro at 37 °C (excitation 485 nm, emission 535 nm), scanning every 2 min for 1 h.

**Cell culture and membrane preparation for DAGL, MAGL assay.** Cell culture and membrane preparation were performed as previously described31. HEK293T cells (American Type Culture Collection, ATCC) were cultured at 37 °C with 5% CO2 in humidified atmosphere with 10% fetal bovine serum. Cells were passaged every 2–3 days by resuspending in medium and feeding them to appropriate confluence. Membranes were prepared from transiently transfected HEK293T cells. One day prior to transfection 10⁶ cells were seeded in a 15 cm petri dish. Cells were transfected by the addition of a 3:1 mixture of polyethyleneimine (60 µg) and plasmid DNA (20 µg) in 2 ml serum-free medium. The medium was refreshed after 24 h, and after 72 h the cells were harvested by suspending them in 20 ml medium. The suspension was centrifuged for 10 min at 1000 rpm, and the supernatant was removed. The cell pellet was stored at −80 °C until use. Cells were homogenized on ice and then resuspended in lysis buffer 1: 20 mM Hepes, 2 mM DTT, 0.25 M sucrose, 1 mM MgCl₂, 2.5 µM β-mercaptoethanol and incubated 30 min on ice. The suspension was subjected to ultracentrifugation (100,000 x g, 30 min, 4 °C, Beckman Coulter, Type T570 rotor) to yield the cytosolic fraction in the supernatant and the membrane fraction in the pellet. The membrane fraction was resuspended in lysis buffer 2 (20 mM HEPES, 2 mM DTT). The protein concentration was determined with Quick Start Bradford assay (Biorad). The protein fractions were

**Data analysis for bias calculations.** The data used for the operation analysis was the data of at least three independent experiments on each assay, all normalized to the effect of 10 µM CP55940. The analysis was used based on van der Westhuizen et al.30 For more details about the discovery of the operational model and its mathematical background, see Black and Leff and Kenakin et al.34,35 See Supplementary Methods for a full step-by-step procedure.

**AEA reuptake inhibition in HaCaT cells.** The uptake of [3H]AEA was measured in intact HaCaT cells (a kind gift of Prof. N.E. Fusetig (German Cancer Research Center, Heidelberg, Germany), that were incubated in PBS at 37 °C with a mixture of AEA [arachidonyl-5,6,8,9,11,12,14,15-3H] (200 Ci mmol–1) and cold AEA (at a final concentration of 400 nM) for 15 min (ref. 56). Control experiments were carried out also at 4 °C and in the presence of the selective AEA reuptake inhibitor OMDM-1 (10 µM). The effect of different ligands on AEA uptake was tested by adding each substance directly to the incubation medium.

**AEA reuptake inhibition in HMC-1 cells and U937 cells.** Compound screening and IC50 determinations for AEA cellular uptake in U937 cells and HMC-1 cells (source: U937 cells were purchased from ATCC, Manassas, VA, USA, HMC-1 cells were a gift of Prof S. Ständer, University of Münster with the permission of the Mayo Foundation, USA) was performed in 96-well format using AquaSili silanized glass vials (Chromat envision 1.1-MTY) (refs 32,57). First, required amount of U937 cells were centrifuged at 100g for 5 min and resuspended in RPMI (37 °C) to a final concentration of 2 x 10⁶ cells ml–1. Then, 250 µl of cell suspension (0.5 x 10⁶ cells per sample) were transferred into the glass vials. After addition of 5 µl vehicle (DMSO) or test compounds dissolved in DMSO at indicated final concentrations (CP55940 and the response of 1 µM CP55940 and the response of

**Human cAMP assay.** cAMP assays were performed with CHO cells stably expressing human CB₁ or human CB₂ receptors22 (source: Discoverx, Revere, MA, USA) using the cAMP-Nano-TRF detection kit (Roche Diagnostics, Penzberg, Germany). Cells were seeded 17–24 h prior to the experiment at a density of 3 x 10⁴ cells per well in a black 96-well plate with flat clear bottom (Corning Costar, NY, USA) in a humidified incubator. The growth medium was exchanged with Krebs Ringer bicarbonate buffer with 1 mMol/l – 3-isobutyl-1-methylxanthine (IBMX), 0.1% fatty acid-free BSA and incubated at 30 °C for 60 min. Agonist was added to a final assay volume of 100 µl and the mixture was incubated for 30 min at 30 °C. The assay was stopped by the addition of 20 µl lysing reagent and shaken for 2 h at room temperature. The time-resolved energy transfer was measured using an LS502 Nanoscan FLT (IOM, Berlin, Germany), equipped with a laser as excitation source. cAMP content was determined from the function of a standard curve spanning from 10 to 0.13 nmol/l–1 cAMP.

**Data analysis of functional assays.** All experimental data were analysed using the nonlinear regression curve fitting program GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA, USA). From displacement assays, pEC⁰ values were obtained by non-linear regression analysis of the displacement curves. The obtained pEC⁰ values were converted into pKᵢ values using the Cheng Prusoff equation to determine the affinity of the ligands (Kᵢ) = 0.33 (CB₁R), 0.10 (CB₂R), (ref. 55). β-Arrestin recruitment and GTP·Y/S curves were analysed by the nonlinear regression option ‘log (agonist or inhibitor) versus response-variable slope’ to obtain potency, inhibitory potency or efficacy values of agonists and inverse agonists (E₅₀, IC₅₀ or Iₗ₅₀, respectively). Basal activity of the cells is set at 0%. For the β-arrestin recruitment assay, all data points were corrected for any background (for example, background luminescence). For the analysis of antagonists, the IC₅₀ was determined in competition with CP55940, the nonlinear regression option ‘log (agonist or inhibitor) versus response’ was chosen. The response of agonists per sample is normalized to the effect of 10 µM CP55940 and the response of antagonists is normalized to effect of the EC₅₀ of CP55940. For the GTP·Y/S assay, agonistic effect is normalized to the effect of 10 µM CP55940. Data shown are the mean ± s.e.m. of at least three independent experiments, each performed in duplicate, unless stated otherwise.
diluted to a total protein concentration of 1 mg ml\(^{-1}\) and stored in small aliquots at \(-80^\circ\text{C}\) until use.

Biochemical DAGL\(\alpha\) activity assay. hDAGL-\(\alpha\) activity was measured by the extent of the hydrolysis of para-nitrophenylbutyrate (PNP-butyrate) by membrane preparations from HEK293T cells (source: Dutch Cancer Institute) transiently transfected with hDAGL-\(\alpha\) (ref. 43). Percentage of inhibition of reference ligands was determined in comparison with an untreated control. Standard assay conditions are as follows: 0.2 U ml\(^{-1}\) of ethanolamine kinase (GK), glycerol-3-phosphate oxidase (GPO) and horseradish peroxidase (HRP) in 10 mM Tris-HCl, pH 7.6 and 1% w/v fatty acid-free BSA. Successively, a mixture of 99.5 nM of AEA and 0.5 mM of [ethanolamine-1-\(^3\)H]-AEA (40–60 Ci mmol\(^{-1}\)) was added to the homogenate (final concentration of 100 nM) and incubated for 10 min at 37\(^\circ\text{C}\) under shaking. After the incubation time, 2 volumes of an ice-cold methanol:chloroform mixture (1:1 v/v) were added to each sample, vigorously vortexed and centrifuged at 10,000g for 10 min at 4\(^\circ\text{C}\) to separate aqueous and organic phases. The aqueous phases were collected and the radioactivity associated with the hydrolysis product [\(^3\)H]-ethanolamine was measured after addition of 3 ml of scintillation cocktail using the Tri-Carb 2100 TR scintillation counter. Data were collected from three independent experiments performed in triplicate and results were expressed as FFAH activity, relative to that in vehicle-treated samples (\(=100\%\)).

COX2 activity. The COX2 activity was assessed using a COX fluorescent inhibitor screening assay kit from Cayman Chemicals. The assay was performed in a final volume of 50 ml in black 384-well non-binding microplates. Tested compounds (at the concentrations of 1 and 5 \(\mu\text{M}\)) or solvent (less \(\leq 1\%\) of the final volume) were pre-incubated at 37\(^\circ\text{C}\) for 30 min with 100 \(\mu\text{g}\) of cell homogenate diluted in assay buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.6 and 1% w/v fatty acid-free BSA). After incubation, the mixture was heated to 70\(^\circ\text{C}\) for 3 min and then immediately cooled on ice and vortexed for 1 min. The samples were centrifuged (16,100 \(\times\) g, 15 min, 4\(^\circ\text{C}\)), vortexed and centrifuged (16,100 \(\times\) g, 15 min, 4\(^\circ\text{C}\)) again. The supernatant was transferred to scintillation vials and 12.5 ml v/v of scintillation cocktail was added. Fluorescence was measured in 5 min intervals for 60 min.

Off-target activity on TRP channels. HEK293 (human embryonic kidney) cells stably over-expressing recombinant human TRPV1 or rat TRPA1, TRPV3, TRPV4 or TRPM8 (source: human embryonic kidney cells were purchased by DSMZ (Germany), TRPV1-HEK-293 cells were a kind gift from John Davis, GlaxoSmithKline, Harlow, UK, the plasmid for TRPV2, as well as TRPV3-HEK-293 and TRPV4-HEK-293 were a kind gift from HB Bloor of Indiana University, the plasmid containing TRPA1-HEK was a kind gift from Sven-Eric Jörndt then at Department of Cellular and Molecular Pharmacology University of California, San Francisco, California, USA and now at Department of Anesthesiology, Duke University School of Medicine, Durham, NC, USA, TRPM8-HEK-293 was a gift from Mario van der Stelt) were grown on 100 mm diameter Petri dishes in monolayers in minimum essential medium supplemented with non-essential amino acids, 10% FBS, 2 mM glutamine, and maintained at 5% CO\(_2\) at 37\(^\circ\text{C}\). Quantitative real-time analysis was carried out to measure TRP gene over-expression in transfected cells (data not shown). On the day of the experiment, cells were loaded with the methyl ester Fluo-4 AM in minimum essential medium (4 \(\mu\text{M}\) in DMSO containing 0.02% Pluronic F-127, Invitrogen), kept in the dark at room temperature for 1 h, washed twice with Tyrode’s buffer (145 mM NaCl, 2.5 mM KCl, 1.5 mM Ca\(_2\)Cl\(_2\), 1.2 mM Mg\(_2\)Cl\(_2\), 10 mM d-glucose, and 10 mM HEPES, pH 7.4), resuspended in the same buffer and transferred (about 70,000 cells) to the quartz cuvette of the spectrofluorimeter (Perkin-Elmer LS580 equipped with PTP-1 Fluorescence Peltier System; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) under continuous stirring. The effects of intracellular Ca\(_{\text{2+}}\) concentration (\(\text{Ca}_{\text{2+}}\)) before and after the addition of various concentration of test compounds was measured by cell fluorescence (\(\text{Ca}_{\text{2+}}\)) at 25\(^\circ\text{C}\). The effects were normalized against the response to ionomycin (4 \(\mu\text{M}\) in each experiment). The increases in fluorescence in wild-type HEK293 cells (that is, not transfected with any construct) were used as baseline and subtracted from the values obtained from transfected cells. Efficacy was defined as the maximum response elicited by the compounds tested in each experiment determined by comparing the effect with the response with 4 \(\mu\text{M}\) ionomycin (Cayman), while the potency of the compounds (\(\text{EC}_{\text{50}}\)) was determined as the concentration required to produce half-maximal increases in [\(\text{Ca}_{\text{2+}}\]). Curve fitting (sigmoidal dose-response variable slope) and parameter estimation were performed with GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA).

Antagonist/desensitizing behaviour was evaluated by adding the test compounds in the quartz cuvette 5 min before stimulation of cells with agonists. In the case of human TRPV1-expressing HEK293 cells the agonist used was capsaicin (0.1 \(\mu\text{M}\), in the case of SR141716A 10 \(\mu\text{M}\) was also used), which was able of eliciting intracellular Ca\(_{\text{2+}}\) with a potency of \(\text{EC}_{\text{50}}\) = 5.3 \(\pm\) 0.4 \(\mu\text{M}\) and efficacy = 78.6 \(\pm\) 0.6%. For TRPV2, the rat TRPV2-HEK-293 cells exhibited a sharp increase in [\(\text{Ca}_{\text{2+}}\]), upon application of lypso-phosphatidylcholine (LPC) 3 \(\mu\text{M}\). The concentration for half-maximal activation was 3.40 \(\pm\) 0.02 \(\mu\text{M}\) and efficacy was 91.7 \(\pm\) 0.5%. In the case of TRPV3, rat TRPV3-expressing HEK-293 cells were first sensitized with the non-selective agonist 2-aminoethoxydiphenyl borate (100 \(\mu\text{M}\)). Antagonist/desensitizing behaviour was evaluated against thymol (100 \(\mu\text{M}\)), which showed an efficacy of 34.7 \(\pm\) 0.1% with a potency of \(\text{EC}_{\text{50}}\) = 84.1 \(\pm\) 1.6 \(\mu\text{M}\).

In the case of rat TRPV4-expressing HEK-293 cells the agonist used was 4\(\mu\text{M}\) 12,13-didecanoyl (4D1D) (1 \(\mu\text{M}\)), which was able of elevating intracellular Ca\(_{\text{2+}}\) with a potency of \(\text{EC}_{\text{50}}\) = 4.60 \(\pm\) 0.07 \(\mu\text{M}\), and an efficacy of 51.9 \(\pm\) 1.7%.

In the case of rat TRPM8-expressing HEK-293 cells, antagonist/desensitizing behaviour was evaluated against icilin at 0.25 \(\mu\text{M}\) and 0.10 \(\mu\text{M}\). For icilin, efficacy was 16.0 \(\pm\) 0.1% and potency \(\text{EC}_{\text{50}}\) = 0.01 \(\mu\text{M}\).

In the case of HEK-293 cells stably over-expressing recombinant rat TRPA1, the effects of TRPA1 agonists are expressed as a percentage of the effect obtained with
100 μM allyl isothiocyanate, which showed a potency of EC50 = 1.41 ± 0.04 μM and a selectivity of 65.9 ± 0.5.

The effect on [Ca2+], exerted by agonist alone was taken as 100%. Data are expressed as the concentration exerting a half-maximal inhibition of agonist-induced [Ca2+], elevation (IC50), which was calculated using GraphPad. All determinations were performed at least in triplicate. Statistical analysis of the data was performed by analysis of variance at each point using ANOVA followed by the Bonferroni’s test.

CEREP panel. Pharmacological profiles of the test compounds were generated. Data show given the percentage of inhibition for binding assays and the percentage of inhibition for enzyme and cell-based assays at a test concentration of 10 μM. The assays were performed at CEREP according to standard procedures described under http://www.cerep.fr/cerep/users/index.asp. For the first compounds being evaluated (WIN55212-2, CP55940, SR141716A, JWH133, HU308, Gp-1a and MSE196204) were obtained from BioreclamationIVT (NY, USA). For human, ultra-pooled (150 USA donors) liver microsomes are purchased to account for the biological variance in vivo. For the microsome incubations, 96 deep well plates are applied, which are incubated at 37 °C on a TECAN (Tecan Group Ltd, Switzerland) equipped with Te-Shake shakers and a warming device (Tecan Group Ltd, Switzerland). The incubation buffer is 0.1 M phosphate buffer pH 7.4. The NADPH regenerating system consists of 30 mM glucose-6-phosphate disodium salt hydrate; 10 mM NADP; 30 mM MgCl2 × 6 H2O and 5 mg ml−1 glucose-6-phosphate dehydrogenase (Roche Diagnostics) in 0.1 M potassium phosphate buffer pH 7.4.

Incubations of a test compound at 1 μM in microsome incubations of 0.5 mg ml−1 plus cofactor NADPH are performed in 96-well plates at 37 °C. After 1, 3, 6, 9, 15, 25, 35 and 45 min 4 μl incubation mixture is taken out in time using a linear fit. The calculated slope is used to determine the intrinsic clearance: Clint (μl min−1 per mg protein) = −slope (min−1) × 1,000/[protein concentration (mg ml)−1]. Data are obtained from single experiments measured with multiple time-points.

Hepatocyte clearance. For animals, hepatocyte suspension cultures are either freshly prepared by liver perfusion studies or prepared from cryopreserved hepatocyte batches. For human, commercially available, pooled (5–20 donors), cryopreserved hepatocytes from non-transplantable liver tissue are used (source: primary, pooled human cryopreserved hepatocytes (Lot ECO) from nontransplantable liver tissues and pooled C57BL6 mouse hepatocytes (Lot PJI)) were purchased from BioreclamationIVT (NY, USA). For the suspension cultures, Nunc U96 PP-0.5 ml (Nunc Natural, 267245) plates are used, which are incubated in a Thermo Forma CellExpert (Wohlen, Switzerland) equipped with shakers from Varimagi Teleshake shakers (Sterico, Wangen, Switzerland) for maintaining cell dispersion. The cell culture medium is William’s media supplemented with Glutamine, antibiotics, insulin, dexamethasone and 10% FCS.

Incubations of a test compound at 1 μM concentration in suspension cultures of 1 Mio cells ml−1 (1 mg ml−1 protein concentration) are performed in 96-well plates and shaked at 900 rpm for up to 2 h in a 5% CO2 atmosphere and 37 °C. After 3, 6, 10, 20, 40, 60 and 120 min 100 μl cell suspension in each well is quenched with 200 μl methanol containing an internal standard. Samples are then cooled and centrifuged before analysis by LC-MS/MS.

Log peak area ratios (test compound peak area/internal standard peak area) are plotted versus time using a linear fit. The calculated slope is used to determine the intrinsic clearance: Clint (μl min−1 per mg protein) = −slope (min−1) × 1,000/[protein concentration (mg ml)−1]. Data are obtained from single experiments measured with multiple time-points.

Plasma protein binding. Pooled and frozen plasma from selected species were obtained from commercial suppliers (The pooled and frozen plasma from human HDPELTA, Lot BRHR1060627) and mouse (MSE196204) were obtained from BioreclamationIVT (NY, USA). The Telfon equilibrium dialysis plate (96-well, 150 μl, half-cell capacity) and cellulose membranes (12–14 kDa molecular weight cutoff) were purchased from HT-Dialysis (Gales Ferry, CT, USA). Both biological matrix and phosphate buffer pH are adjusted to 7.4 on the day of the experiment. The reference substance is diazepam.

The determination of unbound compound is performed using a 96-well format equilibrium dialysis device with a molecular weight cut-off membrane of 12-14 kDa. The equilibrium dialysis device itself is made of Telfon to minimize non-specific binding of the test substance. Compounds are tested in cassettes of 2–5 with an initial total concentration of 1000 nM, one of the compound cassette being the positive control diazepam.

Equal volumes of matrix samples containing substances and blank dialysis buffer (Soerensen buffer at pH 7.4) are loaded into the opposite compartments of each well. The dialysis block is sealed and kept for 5 h at a temperature of 37 °C and 5% CO2 environment in an incubator. After this time, equilibrium will have been reached for the majority of small molecule compounds with a molecular weight of ≤500 Da. Afterward, dialysis membranes are removed and matrix-buffer from one compartment is prepared for analysis by LC-MS/MS. All protein binding determinations are performed in triplicates. The integrity of membranes is tested in the HTDialysis device by determining the unbound fraction values for the positive control diazepam in each well. After an equilibrium, the unbound drug concentration in the biological matrix compartment of the equilibrium dialysis apparatus is the same as the concentration of the compound in the buffer compartment. Thus, the percent unbound fraction (fu) can be calculated by determining the compound concentrations in the buffer and matrix compartments after dialysis as follows: fu% = 100 × buffer conc after dialysis/matrix conc after dialysis. The device recovery is checked by measuring the compound concentrations in the matrix before dialysis and calculating the percent recovery (mass balance). The recovery must be within 80-120% for data acceptance.

P-glycoprotein binding. P-glycoprotein (permeability-glycoprotein, abbreviated as P-gp) also known as multidrug resistance protein 1 (MDR1) is the most studied and characterized drug transporter. The P-gp assay evaluates the ability of test compounds to be transported transcellularly as a P-gp substrate. Incubations of a test compound at 1 μM in microsome incubations of 0.5 mg ml−1 plus cofactor NADPH are performed in 96-well plates at 37 °C. After 1, 3, 6, 9, 15, 25, 35 and 45 min 4 μl incubation mixture is taken out in time using a linear fit. The calculated slope is used to determine the intrinsic clearance: Clint (μl min−1 per mg protein) = −slope (min−1) × 1,000/[protein concentration (mg ml)−1]. Data are obtained from single experiments measured with multiple time-points.

PAMPA. PAMPA (Parallel Artificial Membrane Permeability Assay) is a method which determines the permeability of substances from a donor compartment, through a lipid-embedded artificial membrane into an acceptor compartment. Readout is a permeation coefficient Pf as well as test compound concentrations in donor and acceptor compartments.

A 96-well microtiter plate completely filled with aqueous buffer solutions (pH 7.4/6.5) is covered with a microfilter plate like a sandwich construction. The hydrophilic filter material (Durapore/Millipore; pore size 0.22–0.45 μm) of the first 48 wells (sample) of the filterplate is impregnated with a 1–20% solution of lecinthin in an organic solvent (dodecane, hexadecane, 1,9-decadiene). The filter surface of the remaining 48 wells (reference) is wetted with a small volume (4–5 μl) of a 50% (v/v) methanol/buffer solution. Transport studies were started by the transfer of 100–200 μl of a 250 or 500 μM stock solution on top of the filterplate in the sample and in the reference section, respectively. In general 0.05 M TRIS, pH 7.4 or 0.03 M phosphate, pH 6.5, buffers were used. The maximum DMSO content of the stock solutions was 5%.

Kinetic solubility. The solubility of a test compound in phosphate buffer at pH 6.5 from evaporated DMSO compound stock solution is measured over time, resulting in the kinetic solubility of the compounds.

Triad assay in mice using cumulative dosing procedure. These animal experiments were performed in the lab of Prof Lichtman, Virginia Commonwealth University. Male C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and were between 10 and 12 weeks of age during treatment. The animal protocol for the triad assay was approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. After testing was completed, all mice were humanely euthanized under CO2 asphyxia, followed by rapid cervical dislocation. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals.
In vivo results.

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