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Modulation of L-α-lysophosphatidylinositol/GPR55 MAP kinase signalling by CB_2 receptor agonists: identifying novel GPR55 inhibitors

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Abstract

Background: GPR55 is a lipid-sensing G protein-coupled receptor that is activated by the endogenous lipid L-α-lysophosphatidylinositol (LPI) and can be modulated by certain cannabinoid ligands.
Methods: In this study we investigated the GPR55 activity of four synthetic CB_2 receptor agonists using the AlphaScreen® SureFire® assay.
Results: Here we show that the CB_2 receptor-selective agonists HU-308, HU-433 and HU-910 do not promote GPR55-mediated ERK1/2 phosphorylation up to a concentration of 3 μM. However, LPI-induced ERK1/2 phosphorylation is inhibited by the (–)-enantiomer of HU-308, designated HU-433, whereas HU-308 has no effect on LPI activity. The carboxylic analogue of HU-910, designated HU-914, potently inhibits LPI-induced ERK1/2 phosphorylation; however, HU-914 was less effective, with potential biphasic effects.
Conclusions: This structure-activity-relationship study has identified novel ligands which act both as CB_2 receptor agonists and GPR55 modulators and related compounds that lack GPR55 activity.

Keywords: AlphaScreen; cannabinoids; CB_2 receptor-selective agonists; endocannabinoids; ERK1/2; GPR55.

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Introduction

The diverse physiological roles of GPR55 and its potential involvement in the pathophysiology of various medical conditions have promoted much research into its pharmacology, with a view to identifying selective ligands that will help define its function [1–8]. GPR55 is activated by the endogenous lipid L-α-lysophosphatidylinositol (LPI), by selected cannabinoids and also by newly identified synthetic molecules resulting from high-throughput screenings [7, 9–12]. In particular, this receptor is thought to be an important new therapeutic target regulating cancer cell function. For example, GPR55 is highly expressed in certain tumours and its activation by LPI increases cancer cell migration, invasion and proliferation [5, 13, 14]. In addition, increased levels of LPI have been found in the blood of cancer patients [15]. Based on these results, considerable attention is now being paid to the identification of GPR55 antagonists [9, 16]. Other potential clinical uses for GPR55 inhibitors have emerged from studies showing that activation of GPR55 contributes to inflammation and enhances neuropathic pain [17]. Furthermore, GPR55-deficient mice demonstrate increased bone density, suggesting that GPR55 regulates bone mass and ligands which inhibit GPR55 activity may have utility in treating osteoporosis [18].

Interestingly, inhibition of GPR55 produces effects that often resemble those of cannabinoid CB_2 receptor activation [18, 19] and there is also evidence of a functional cross-talk between these two receptors [20]. Therefore, it is important to re-evaluate the pharmacology of CB_2 receptor-selective agonists and test for potential interactions with GPR55. We have previously published data with selected CB_2 receptor-selective agonists, where we found that GW405833 behaves as a partial agonist of the human GPR55 (hGPR55) and enhances the LPI-induced ERK1/2 phosphorylation at a concentration which alone has no effect on pERK [9]. Conversely, we have found that JWH-133 reduces the hGPR55-mediated ERK1/2 phosphorylation and at concentrations of 1–3 μM inhibits the LPI-induced response. On the other hand, (E)-β-caryophyllene and AM1241 have no effect up to concentrations of 10 μM and do not significantly alter

LPI-induced GPR55 responses. Here, we extended upon these findings and evaluate the GPR55 activity of synthetic CB_2 receptor-selective ligands. We have used the same experimental methods as previously described by Anavi-Goffer et al. [9] where GPR55 activity is profiled using HEK293 cells stably expressing HA-tagged human GPR55 [9, 21]. Focusing on the phosphorylation of ERK1/2 protein by GPR55, as this signalling pathway is one of the main effectors downstream of GPR55 stimulation, we compared the actions of HU-308 with those of its (–)-enantiomer (HU-433; Figure 1), and that of HU-910 with its carboxylic analogue (HU-914; Figure 1), all of which structurally resemble Δ^9-THC, the main psychoactive phytocannabinoid of cannabis.

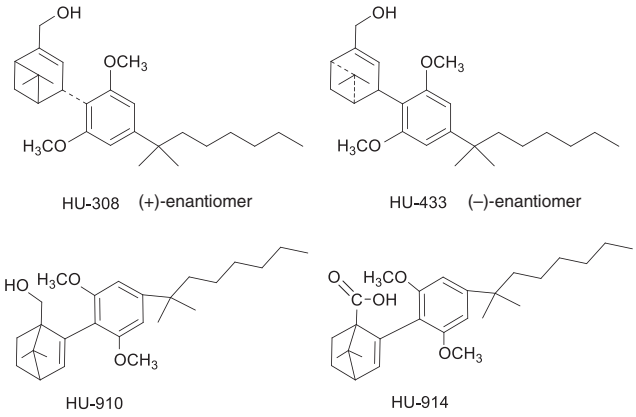

Figure 1: Illustration of the chemical structures of HU-308 and its (–)-enantiomer, designated HU-433.
Illustrated are also the compound HU-910 and its carboxylic acid analogue, designated HU-914.

Materials and methods

Materials

G-418 from PAA (Cambridge, UK), DMEM/F12, DMEM, Newborn calf serum and Pen/Strep solution were obtained from Fisher Scientific (Loughborough, UK). L-Glutamine, LPI and all the other chemicals were obtained from Sigma-Aldrich (Dorset, UK). An AlphaScreen® SureFire Phospho-ERK Kit (catalogue TGRES10K) was obtained from PerkinElmer (Bucks, UK). HU-308 was obtained from Tocris (Bristol, UK). The following compounds were synthesised by Raphael Mechoulam, The Hebrew University of Jerusalem, Israel, and kindly gifted for this study: HU-910 [22], HU-914 (unpublished data, displacement of [^{3}H]CP55940 yielded a Ki value of 1500 nM for the CB_2 receptor; the Ki value was not determined for the CB_1 receptor; presumably this value is in the same range as the Ki value of HU-910 for the CB_1 receptor) and HU-433 [23].

Cell culture

The preparation of HEK293 stably expressing the tagged human GPR55 receptor (hGPR55-HEK293) has been published previously by Henstridge et al. [21] and used to characterise the effect of selected cannabinoids at GPR55 by Anavi-Goffer et al. [9]. Briefly, the cells were maintained in Dulbecco's modified Eagle's medium DMEM/F12 supplemented with 10% newborn calf serum, 0.5 mg/mL G-418, 60 units penicillin, 60 µg streptomycin and 2 mM L-glutamine at 37 °C and 5% CO_2.

ERK1/2 MAP-kinase phosphorylation assay

The assay was conducted as previously described by Anavi-Goffer et al. [9]. Briefly, cells were plated onto 96-well plates at a density of 40,000 cells/well and serum-starved for 48 h in the DMEM/F12 medium supplemented with G-418 and 2 mM L-glutamine. Cells were assayed in the DMEM/F12 medium containing L-glutamine and incubated for 20 min at 37 °C in a humidified atmosphere. Drugs were dissolved at a concentration of 10 mM in DMSO. LPI was stored at –80 °C for up to 3 months. Drugs were tested in the absence of LPI at a final concentration of 0.1% DMSO or in the presence of LPI at a final concentration of 0.2% DMSO.

AlphaScreen® SureFire® ERK assay

The medium was removed and cells were lysed with lysis buffer (AlphaScreen® SureFire® ERK kit). Samples of 4 µL were incubated with 7 µL of mixture containing 1 part donor beads:1 part acceptor beads:10 parts activation buffer:60 parts reaction buffer. Plates were incubated at 23–25 °C and read using AlphaScreen® settings by an Envision system (PerkinElmer).

Statistical analysis

Results are presented as means and variability as SEM or 95% confidence limits (CL) of the percent stimulation of phosphorylated ERK1/2 above the basal level (in the presence of vehicle) [9]. Data were analysed using nonlinear analysis of the log agonist-vs.-response curve using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). The results of this analysis were presented as E_{max}±SEM and pEC_{50}±SEM ($logEC_{50}$) or EC_{50}±95% CL (where appropriate). Data were presented as 'pERK' stimulation as percent of LPI. The statistical significance of E_{max}±SEM or $logEC_{50}$±pEC_{50} was determined with an unpaired Student's t-test.

Results

Effect of CB_2 receptor agonists on hGPR55-mediated ERK1/2 phosphorylation

In HEK293 cells expressing hGPR55, LPI produced a maximal stimulation of 125.8%±9.58 (E_{max}) with an EC_{50} of 1.04 µM (0.52–2.09) (sum of data from Figure 2A–D;

p<0.0001, n=10 each in duplicate, not shown). The maximal stimulation with 0.1% DMSO was not significantly different from that with 0.2% DMSO [9]. In untransfected HEK293 cells, no stimulation of phosphorylated ERK1/2 was detected at different concentrations of LPI ranging from 30 nM up to 1 μM, at any given concentration of DMSO [9]. These results are in agreement with a previous report for the lack of effects of LPI up to 10 μM on ERK1/2 stimulation in untransfected HEK293 cells [24]. It should be noted that cell lines are highly heterogeneous and can respond differently to the same ligands. Nevertheless, our results for ERK1/2 phosphorylation in the HEK293-hGPR55 cell line are in agreement with previous studies, e.g. for SR141716A and cannabidiol (CBD) [5, 6, 9, 11]. HU-308 had no effect on hGPR55-mediated ERK1/2 phosphorylation up to a concentration of 3 μM [9]. Similar to HU-308, the compounds HU-910, HU-914 and HU-433 also had no effect up to a concentration of 3 μM (Figure 2). However, HU-308 had a significant effect at a concentration of 10 μM, producing 20.12%±6.26 stimulation of ERK1/2 phosphorylation (Figure 2A; p<0.05, Table 1) [9], whereas HU-433, the (–)-enantiomer of HU-308, and HU-910 had no effect on the stimulation of ERK1/2 phosphorylation at this concentration (Figure 2B and D). The effect of HU-914, the carboxylic analogue of HU-910, was 23.54%±27.82 stimulation; however, this was not significantly different from DMSO-treated cells. Data are summarised in Table 1.

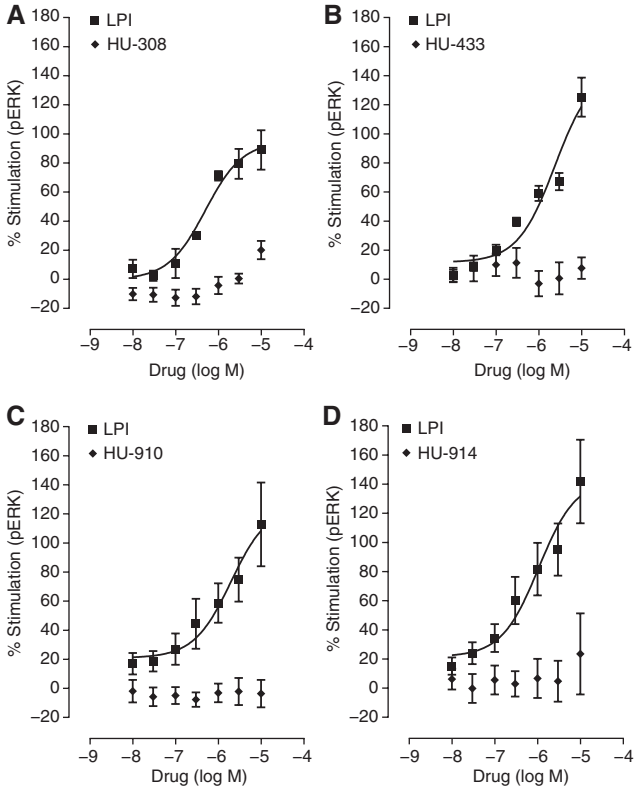

Figure 2: The effect of CB_2 receptor agonists on ERK1/2 phosphorylation in hGPR55-HEK293 cells.
Mean log concentration-response curves of percent stimulation of ERK1/2 phosphorylation by (A–D) LPI (n=3–4), (A) HU-308 (n=3), (B) HU-433 (n=4), (C) HU-910 (n=3) or (D) HU-914 (n=4) after 20 min of stimulation at 37 °C. Each symbol represents the mean percentage change in bound phosphorylated ERK1/2±SEM over the basal level. Each independent experiment was performed in duplicate. Data are summarised in Table 1.

Effect of CB_2 receptor agonists on LPI-induced ERK1/2 phosphorylation: implications for negative allosteric modulation

In the following experiments, LPI produced a maximal stimulation of 118.7%±9.28 (E_{max}) with an EC_{50} of 1.25 μM (0.69–2.27) (sum of data from Figures 3 and 4; p<0.0001, n=15 each in duplicate, not shown). These results are not significantly different from the results for Figure 2. Of the tested compounds, HU-308 had no effect on LPI-induced ERK1/2 phosphorylation at concentrations of 1 and 3 μM (Figure 3A and B, Table 2). In comparison, HU-433, the (–)-enantiomer of HU-308, had no effect on the efficacy, potency or bottom of the curve of LPI-induced ERK1/2 phosphorylation at a concentration of 10 nM (Figure 3D), but HU-433 significantly reduced the E_{max} of LPI of 80.12%±5.42 to 49.67%±6.72 at a concentration of 1 μM, leading to 38% inhibition of LPI-induced ERK1/2 phosphorylation (p<0.05; Figure 3E, Table 2).

HU-914 had a biphasic effect. It had no effect on the efficacy, potency or bottom of the curve of LPI-induced ERK1/2 phosphorylation at concentrations of 1 μM and 10 μM (Figure 4A and C). In contrast, at a concentration of 3 μM, HU-914 significantly reduced the E_{max} of LPI of 151.5%±41.78 to 29.43%±6.59 (p<0.05; Figure 4B). At this concentration, HU-914 also significantly reduced the bottom of the curve for LPI from 22.46%±8.62 to (–)

Table 1: CB_2 receptor agonist-mediated stimulation of ERK1/2 phosphorylation in hGPR55-HEK293 cells.

CB_2 receptor agonists	EC_{50}, μM±95% CL	% Stimulation at 10 μM
HU-308	NA	20.12±6.26[a]
HU-433	NA	7.69±7.42
HU-910	NA	–3.63±9.52
HU-914	NA	23.54±27.89

Cells were treated with each drug for 20 min at 3 7 °C. Final concentration of DMSO was 0.1%–0.2%. [a]p<0.05 one-sample t-test. NA, not applicable.

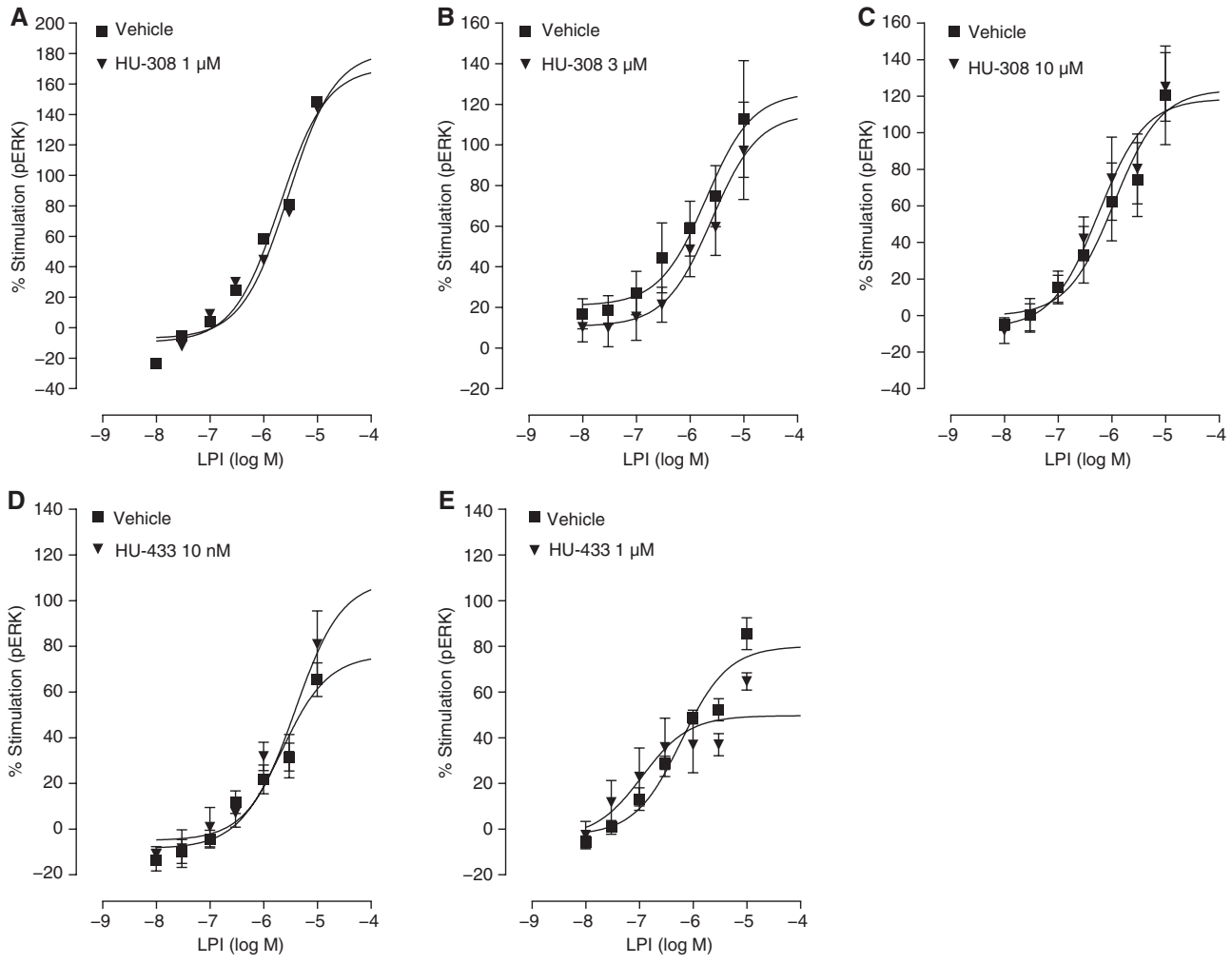

Figure 3: Effect of HU-308 and HU-433 on ERK1/2 phosphorylation in hGPR55-HEK293 cells.
Mean log concentration-response curves of ERK1/2 phosphorylation after 20 min of stimulation at 37 °C, the effect of LPI in the presence or absence of (A) 1 μM HU-308 (n=2) or (B) 3 μM HU-308 (n=3) or (C) 10 μM HU-308 (n=3). The effect of LPI in the presence or absence of (D) 10 nM HU-433 (n=3) or (E) 1 μM HU-433 (n=3). HU-308 did not affect LPI-induced ERK1/2 phosphorylation at the indicated concentrations. HU-433 at 1 μM significantly ($p<0.05$) inhibited the maximal efficacy of LPI-induced ERK1/2 phosphorylation. Each symbol represents the mean percentage change in bound phosphorylated ERK1/2±SEM over the basal level. Each independent experiment was performed in duplicate. Data are summarised in Table 2.

Table 2: The effect of CB_2 receptor agonists on LPI-mediated stimulation of ERK1/2 phosphorylation in hGPR55-HEK293 cells.

CB_2 receptor agonists	LPI±drug	EC_{50}, μM	pEC_{50}±S.E.	% E_{max}±S.E.	% Inhibition
HU-433	Vehicle	0.60	6.22±0.13	80.12±5.42	
	HU-433 1 μM	0.11	6.95±0.40	49.67±6.72[a]	38%
HU-914	Vehicle	1.43	5.85±0.27	155.1±27.87	
	HU-914 1 μM	0.55	6.26±0.26	151.0±19.27	3%
HU-914	Vehicle	3.22	5.49±0.38	151.5±41.78	
	HU-914 3 μM	0.42	6.37±0.29[b]	35.62±7.52[a]	76%
HU-910	Vehicle	3.84	5.4±0.36	188.9±52.22	
	HU-910 3 μM	0.32	6.48±0.43	70.85±12.96	62%

Cells were co-treated with LPI in the presence or absence of a given drug at 1 μM or 3 μM for 20 min at 37 °C. Final concentration was 0.2% DMSO.% Inhibition is percentage inhibition of E_{max} relative to LPI (100%). [a]$p<0.05$ vs. LPI in each experiment. [b]$p<0.001$ vs. LPI in each experiment.

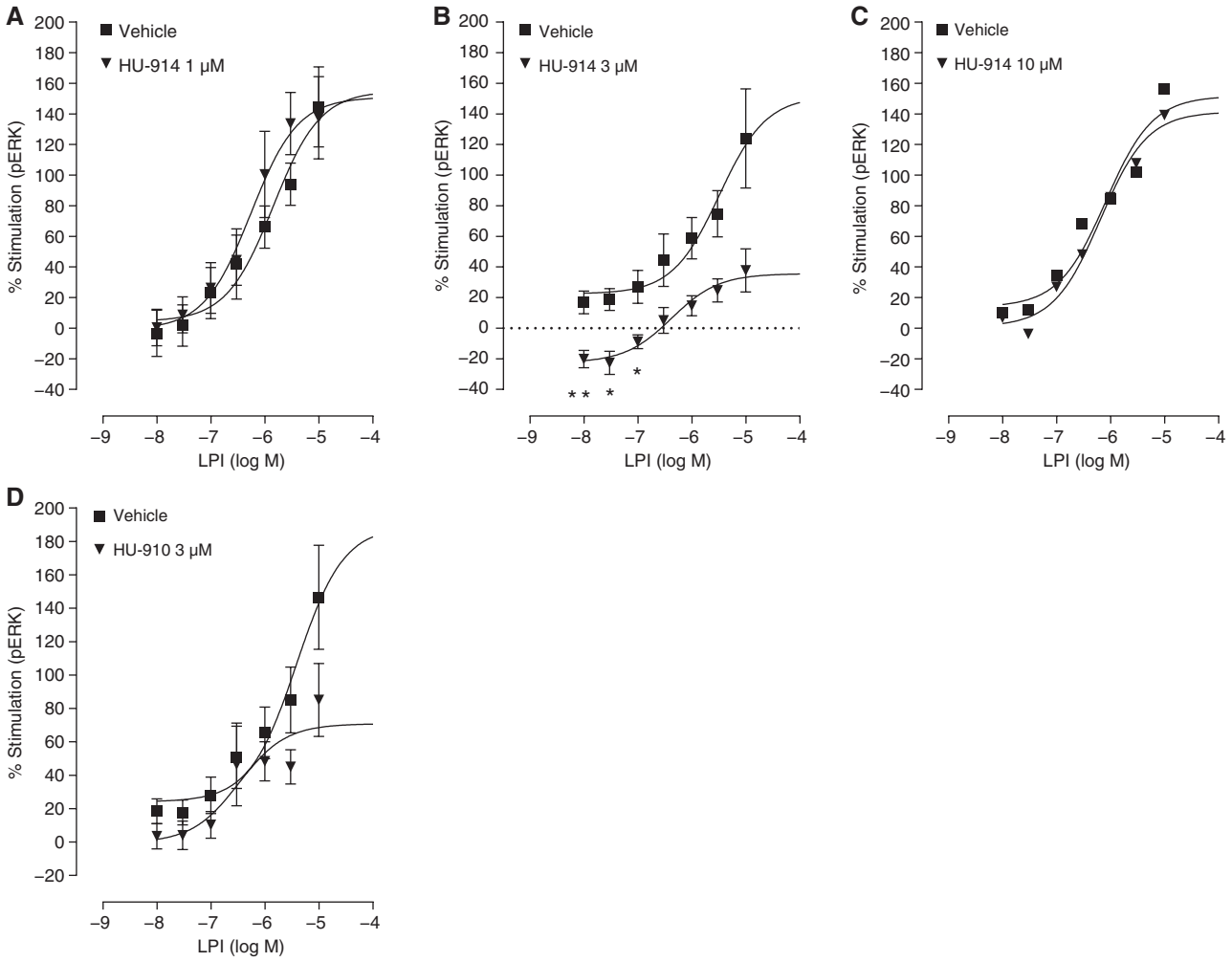

Figure 4: Effect of HU-914 and HU-910 on ERK1/2 phosphorylation in hGPR55-HEK293 cells.
Mean log concentration-response curves of ERK1/2 phosphorylation after 20 min of stimulation at 37 °C, the effect of LPI in the presence or absence of (A) 1 μM HU-914 (n=3), (B) 3 μM HU-914 (n=3) or (C) 10 μM HU-914 (n=2). (D) The effect of LPI in the presence or absence of 3 μM HU-910 (n=3). HU-914 at 3 μM significantly ($p<0.05$) inhibited the maximal efficacy of LPI-induced ERK1/2 phosphorylation and significantly reduced basal pERK levels, *$p<0.05$, **$p<0.01$, one-sample t-test. HU-910 showed a tendency towards the inhibition of LPI-induced ERK1/2 phosphorylation, but this inhibition did not reach a significant level. Each symbol represents the mean percentage change in bound phosphorylated ERK1/2±SEM over the basal level (n=3). Each independent experiment was performed in duplicate. Data are summarised in Table 2.

24.09%±6.57 ($p<0.05$; Figure 4B). A similar reduction of the bottom of the curve has been reported for other cannabinoids by Anavi-Goffer et al. [9] using the MAP kinase assay. This reduction suggests that there is negative cooperative activity, as opposed to positive cooperativity seen with other allosteric modulators [25]. We then tested the effect of HU-910 at a concentration of 3 μM. At this concentration there was a tendency towards inhibition of LPI-induced responses, although this effect did not reach a significant level (Figure 4D; 62% inhibition; $p=0.09$, Table 2). HU-910 had no effect on the potency or the bottom of the curve of the LPI-induced GPR55 response.

Discussion

The pharmacology of CB_2 receptor-selective agonists has been extensively investigated against the classical CB_1 and CB_2 cannabinoid receptors (reviewed in [26]). We have previously reported the effects of selected CB_2 receptor-selective agonists on hGPR55-mediated ERK1/2 phosphorylation and on LPI-induced ERK1/2 phosphorylation of hGPR55 [9]. Here we evaluated the activity of novel analogues of HU-308, a CB_2 receptor-selective agonist, at the hGPR55. Previous studies reported that the analogues HU-433 and HU-910 are also selective agonists of

the CB_2 receptor [22, 23]. However, it appears that HU-914 is a low-potency CB_2 receptor ligand and not a selective ligand of the cannabinoid receptors. We have tested these compounds in the presence or absence of LPI, an endogenous agonist of GPR55. We found that HU-308, HU-433 and HU-910 have no effect on hGPR55-mediated ERK1/2 phosphorylation up to 3 μM. However, a high concentration of 10 μM HU-308 stimulates the GPR55 receptor in the absence of LPI.

Under normal physiological or pathological conditions, GPR55 is likely to be tonically activated by circulating levels of LPI. In the view of this idea, we tested the ability of these compounds to modulate responses to LPI. HU-308 is an anti-inflammatory agent that promotes osteoclastogenesis among its other actions [26–28]. As previously reported, HU-308 had no effect on LPI-induced ERK1/2 phosphorylation [9]. Here we show that its (+)-enantiomer, designated HU-433, produces a significant inhibition of LPI-induced ERK1/2 phosphorylation. Smoum et al. [23] reported that, first, compared with HU-308, HU-433 has reduced affinity to the CB_2 receptor (about 30 times lower), though it has been suggested that the binding pocket of HU-433 at the CB_2 receptor is not identical to that of HU-308. The altered binding configuration within the binding pocket may lead to conformational changes that affect the displacement of the selected radioligand, leading to apparent reduced affinity; secondly, HU-433 is also less potent and less efficacious than HU-308 in the [^{35}S]GTPγS binding assay; thirdly, like HU-308, HU-433 also enhances osteoblast proliferation and inhibits the differentiation of osteoclast although it is the stereoisomer of HU-308; fourthly, both ligands demonstrate a biphasic effect of the stimulation of osteoblast proliferation, but surprisingly HU-433 is more potent than HU-308. Specifically, the concentration of the peak effect of HU-433 is around 1000 times lower than that of HU-308, though HU-433 is less efficacious at the stimulation of the CB_2 receptor. Integrating these findings with our results suggests that there might be an additional mechanism for the actions of HU-433 on bone mass regulation by modulating GPR55 activity. Yet, the lack of response to HU-433 or HU-308 by osteoblasts derived from CB_2 receptor knockout mice questions this hypothesis [23]. This controversy may be explained by putting forward the following hypothesis: it is possible that there is a cross-talk between the CB_2 receptor signalling and GPR55 signalling, but CB_2 receptor signalling orchestrates this interaction in cultured osteoblasts. Indeed, two groups have demonstrated a cross-talk between GPR55 signalling and CB_2 receptor signalling. Balenga et al. [20] have shown that GPR55 signalling can enhance or inhibit CB_2 receptor-mediated responses in a manner that is dependent on the signalling pathway, demonstrating a functional selectivity for GPR55 signalling. Small GTPases, such as Rac2 and Cdc42, have been suggested to mediate this GPR55-CB_2 receptor cross-talk in a model system for migration of human neutrophils [20]. In support of this view, we have previously proposed another cross-talk mechanism, downstream to GPR55, for the interaction of ROCK and Raf signalling [9]. As yet, a cross-talk between GPR55 signalling and CB_2 receptor signalling remains to be investigated in cultured osteoblasts.

It has been reported that HU-910 reduces in a dose-dependent manner the levels of transaminase alanine amino-transferase (ALT) and aspartate amino-transferase (AST) in the serum of animals with hepatic ischaemia/reperfusion injury [22]. Interestingly, pre-treatment with SR144528, a CB_2 receptor-selective antagonist, did not completely prevent this effect of HU-910 on ALT and AST. These results suggest that the effect of HU-910 on ALT and AST levels in this model system for hepatic injury is mainly mediated by the CB_2 receptor but points to the contribution of another pharmacological system to this effect. Recently, GPR55 has been detected in hepatic tissue of human subjects [1]. Our finding of an inhibition of LPI-induced GPR55 activation suggests that GPR55 may be a possible candidate for the residual effect of HU-910 on indicators of liver cellular damage. In support of this view, pre-treatment with the CB_1 receptor-selective antagonist SR141716A was shown to reduce these indicators in the absence of HU-910 [22]. However, we have previously reported that like HU-910, SR141716A inhibits the LPI-induced ERK1/2 phosphorylation of GPR55 [9, 11]. Collectively, these studies suggest that the effect of SR141716A on selected indicators of liver cellular damage could possibly be mediated by GPR55. In support of the effect of HU-910 is the effect of HU-914. Similar to HU-910 but more efficaciously, HU-914 significantly inhibited the LPI-induced ERK1/2 phosphorylation at the same concentration (3 μM), which had no effect on its own on GPR55-induced stimulation of ERK1/2 phosphorylation. This points to the importance of the R group within this molecule for GPR55 inhibition and suggests that the carboxylic group enhances receptor inhibition. However, we noted that the effect of HU-914 was not in a concentration-dependent manner. The biphasic effects of cannabinoids are well documented. These effects have been shown to affect selected behavioural paradigms including locomotor activity [29], feeding [30] and anxiety [31]. Like CBD, HU-914 has two chiral centres; hence there are two enantiomers and altogether four possible isomers. Therefore, it is possible that the potential biphasic effect on LPI responses may be due to the relative level

of each isomer of HU-914 under the current experimental conditions, i.e. it is possible that the inhibitory effects of HU-914 on LPI-induced ERK1/2 phosphorylation of GPR55 are contributed by a more potent 'inverse agonist/antagonist' isomer while increasing the concentration of HU-914 to 10 μM contributed to the actions of a putative 'agonist' isomer that overrides the inhibitory actions of the 'inverse agonist/antagonist' isomer at GPR55. Further investigation will be required in order to fully characterise HU-910/HU-914 isomers.

In summary, the structure-activity-relationship studies presented here together with our previous analysis [9] suggest that selected CB_2 receptor agonists such as HU-308, (E)-β-caryophyllene and AM1241 have no effect on LPI-induced GPR55 signalling, whereas other CB_2 receptor-selective agonists, such as GW405833, can enhance LPI-induced GPR55 signalling. In contrast, HU-433 inhibits LPI-induced GPR55 signalling. This study also points to the structure of HU-914, a low-potency, non-selective cannabinoid ligand, that efficaciously inhibits GPR55 signalling. Our study further directs the development of selective ligands to GPR55 and has identified novel ligands which modulate both the CB_2 receptor and GPR55 activity. Clearly additional validation of these ligands in both non-recombinant systems and against mammalian GPR55 orthologs will be an important step in demonstrating their utility.

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