MODULATION OF L-α-LYSOPHOSPHATIDYLINOSITOL /GPR55 MAP KINASE SIGNALLING BY CANNABINOIDS

Sharon Anavi-Goffer1,4*, Gemma Baillie1, Andrew J. Irving2, Jürg Gertsch3, Iain R. Greig1, Roger G. Pertwee1, Ruth A. Ross1

1School of Medical Sciences, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen, AB25 2ZD, UK; 2Division of Medical Sciences, Ninewells Hospital and Medical School, University of Dundee, DD1 9SY, UK; 3Institute of Biochemistry and Molecular Medicine, University of Bern, Bühlstrasse 28, CH-3012, Bern, Switzerland; 4Departments of Behavioral Sciences & Molecular Biology, Ariel University Center of Samaria, Israel, 40700

Running title: Pharmacology of GPR55 ligands

Address correspondence to: Sharon Anavi-Goffer, Departments of Behavioral Sciences & Molecular Biology, Ariel University Center of Samaria, Israel, E-mail: s.goffer@ariel.ac.il

Background: The endogenous L-α-lysophosphatidylinositol activates GPR55.

Results: Structural analogues of SR141716A act both as agonists alone and as inhibitors of LPI. Certain CB2 receptor agonists also modulate GPR55 activity.

Conclusion: Certain cannabinoids can both activate GPR55 and attenuate LPI-mediated pERK activation. This has mechanistic implications for the antinociceptive effects of certain CB2 agonists.

Significance: Cannabinoid ligands have complex interactions with the LPI/GPR55 signalling system.

SUMMARY

GPR55 is activated by L-α-lysophosphatidylinositol (LPI) but also by certain cannabinoids. In this study we investigated the GPR55 pharmacology of various cannabinoids including analogues of the CB1 receptor antagonist Rimonabant®, CB2 receptor agonists and Cannabis Sativa constituents. To test ERK1/2 phosphorylation, a primary downstream signalling pathway that conveys LPI-induced activation of GPR55, a high-throughput system was established using the AlphaScreen® SureFire® assay. Here we show that CB1 receptor antagonists can act both as agonists alone and as inhibitors of LPI signalling under the same assay conditions. This study clarifies the controversy surrounding the GPR55 mediated actions of SR141716A: some reports indicate the compound to be an agonist, some report antagonism. In contrast, we report that the CB1 ligand GW405833 behaves as a partial agonist of GPR55 alone and enhances LPI signalling. GPR55 has been implicated in pain transmission, thus our results suggest that this receptor may be responsible for some of the antinociceptive actions of certain CB2 receptor ligands. The phytocannabinoids Δ(9)-tetrahydrocannabivarin (THCV), cannabidivarin (CBDV) and cannabigerovarin (CBGV) are also potent inhibitors of LPI. These Cannabis Sativa constituents may represent novel therapeutics targeting GPR55. The physiological roles of GPR55 and its possible involvement in the pathophysiology of medical conditions are emerging. Studies in mice lacking GPR55 have reported a reduction in inflammatory and neuropathic pain (1) and these mice have increased bone mass (2). Subsequently, studies have established that the endogenous lysophospholipid, LPI activates GPR55 (3-10). In certain cancer cell lines, GPR55 is highly expressed and LPI mediates increased cell migration, invasion and proliferation (3,8,11). Moreover, increased circulating levels of LPI have been found in cancer patients and are associated with a poor prognosis (12). These observations suggest that modulation of GPR55 may have therapeutic implications for the treatment of pain, bone diseases and cancer.

To date, certain arylpyrazole CB1 receptor antagonists such as Rimonabant® (also known as SR141716A or Acomplia®) and AM251 have been reported to be GPR55 agonists (5,13,14). However, other groups have
suggested that Rimonabant® is a GPR55 antagonist (6,8). Furthermore, reports on the behaviour of Δ⁹-THC at GPR55 are also inconsistent. Lauckner et al. (2008) (6) have shown that Δ⁹-THC is a GPR55 agonist capable of stimulating calcium release while Kapur et al. (2009) (5) did not detect β-arrestin mediated activation of GPR55 with this phytocannabinoid. Another cannabis constituent, cannabidiol (CBD), is reported to be an antagonist of GPR55 (15). The GPR55 pharmacology of many other Cannabis Sativa constituents has still to be investigated. Furthermore, the pharmacology of various CB₂ receptor-selective ligands at GPR55 has not been investigated. This is important because CB₂ ligands are antinociceptive; a characteristic that may be shared by GPR55 ligands.

Activation of GPR55 by LPI, but also by certain cannabinoïd ligands, initiates multiple signalling pathways distinct from those initiated by cannabinoïd CB₁ and CB₂ receptors. GPR55 is predominantly coupled to Gα₁₂/₁₃ leading to activation of small G proteins (15). It has also been suggested to couple to Gαq to promote the activation of phospholipase C and the increase of intracellular calcium release from inositol triphosphate receptor (IP₃R)-gated stores (6,9). LPI-induced activation of GPR55 has been shown to lead to the recruitment of multiple nuclear transcription factors. Among these factors, the most investigated is ERK1/2 (5-9,11,15-17). Other nuclear transcription factors recruited by GPR55 activation are NFAT (9,13), CREB, NF-κB (4,9,13), p38 MAP-kinase (18) and Akt (8,11).

Here we report on the GPR55 pharmacology of arylpyrazole analogues, CB₂ agonists and a number of Cannabis Sativa constituents (for structures see Table 3). We show that arylpyrazole analogues act both as agonists alone and as inhibitors of LPI signalling. The compounds significantly decrease in the Eₘₐₓ value for the GPR55 endogenous agonist, LPI, which is characteristic of a non-competitive mode of action; this may suggest allostery. These results provide a possible explanation for the controversy surrounding the pharmacology of certain ligands at GPR55, which have been reported, by different groups, to behave as both agonist and antagonists. Here we demonstrate for the first time that a single ligand can display the both behaviours in the same assay. Furthermore, we show that certain CB₂ receptor-selective agonists also act as antagonists of GPR55; this may have implications for the mechanism of action underlying the reported antinociceptive actions of these compounds (19).

**EXPERIMENTAL PROCEDURES**

*Materials.* Cannabis constituents Δ⁹-THC, Δ⁹-THCV, (-)CBD, CBDV, CBG, CBGA, CBGV were supplied by GW Pharmaceuticals and SR141716A was from Sanofi-Aventis (Montpellier, France). (-)CBD, AM251, AM281, CP55940, WIN55212-2 were from Tocris Cookson (Avonmouth, UK). PLX-4720 was from Selleck, Houston, USA. ABD824 was synthesised using similar methodology to that previously described for SR141716A (IRG, University of Aberdeen). BCP ((E)-beta-caryophyllene) was gifted by Prof Gertsch (Institute of Biochemistry and Molecular Medicine, Switzerland). PD98059 from Cayman (USA). G-418 from PAA (UK), DMEM/F12, DMEM, Newborn calf serum and Pen/Strep solution were obtained from Fisher (UK). L-glutamine, LPI, LY294002 and Y27632 and all the other chemicals were obtained from Sigma-Aldrich (Dorset, UK). AlphaScreen® SureFire Phospho-ERK Kit (catalogue TGRES10K) was from PerkinElmer (Bucks, UK).

*Cell culture.* Untransfected HEK293 cells were maintained in DMEM containing 2 mM L-glutamine medium supplemented with 10% fetal bovine serum. The preparation of HEK293 stably expressing the tagged-human GPR55 receptor (hGPR55-HEK293) has been published previously by Henstridge et al. (2009) (4). Briefly, the GPR55 receptor was tagged with a triple hemagglutinin epitope (HA) at the N terminus (3xHA-GPR55), preceded by the signal sequence from the human growth hormone (HGH; residues 1–33) and subcloned into pcDNA 3.1 vector. 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\textsubscript{2}. Transfected cells and untransfected cells were plated on the same plates for comparison.

**ERK1/2 MAP-kinase phosphorylation assay.** For experimental studies of ERK1/2 MAP-kinase phosphorylation, cells (40,000 cells/well) were plated onto 96 well plates and serum-starved for 48 h. hGPR55-HEK293 cells were serum-starved in DMEM/F12 medium supplemented with G-418 and 2 mM L-glutamine. HEK293 cells were serum-starved in DMEM/F12 medium supplemented with 2 mM L-glutamine. Cells were assayed in DMEM/F12 medium containing L-glutamine and incubated for 20 min or 60 min at 37°C in a humidified atmosphere. Drugs were dissolved in DMSO and stocks at a concentration of 10 mM were kept at -20°C. LPI was stored at -80°C for up to three 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, unless stated otherwise. At the end of the assay, the medium was removed and cells were lysed with lysis buffer supplied in the AlphaScreen\textsuperscript{®} SureFire\textsuperscript{®} ERK kit.

**AlphaScreen\textsuperscript{®} SureFire\textsuperscript{®} ERK Assay.** The assay was performed in 384 well white Proxiplates according to the manufacturer instructions. Briefly, 4 µl samples 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 room temperature and read with the Envision system (PerkinElmer) using AlphaScreen\textsuperscript{®} settings.

**Analysis.** Raw data were presented as ‘Envision units’. Basal level was defined as zero. Results were 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). Data were analysed using nonlinear analysis of log agonist versus-response curve using GraphPad Prism 5.0 (GraphPad, San Diego, CA). The results of this analysis were presented as E\textsubscript{max} ± SEM and pEC\textsubscript{50} ± SEM (logEC\textsubscript{50}) or EC\textsubscript{50} ± 95% CL (where appropriate). In graph 1C, for each kinase inhibitor, the values for percent stimulation of phosphorylated ERK1/2 were normalized to the mean value produced by 10 µM LPI (in the presence of vehicle) in matched experiments. Curves of LPI-induced response were not different between experiments; therefore data were pooled. Data were presented as ‘pERK’ stimulation as percent of LPI. The statistical significance of E\textsubscript{max} ± SEM, or logEC\textsubscript{50} ± pEC\textsubscript{50} was determined with an unpaired Student’s t-test (95% confidence interval). When curves could not be fitted on a nonlinear analysis of log agonist versus-response, the statistical significance of the stimulation was determined with an unpaired Student’s t-test at each specific concentration. Results were considered significant only when F-test comparing the variance was not significantly different.

**RESULTS**

**Studying the pharmacology of GPR55 using the AlphaScreen\textsuperscript{®} SureFire\textsuperscript{®} pERK1/2 assay.** The phosphorylation of ERK1/2 protein has been reported as one of the main signalling pathways initiated upon stimulation of the GPR55 receptor; therefore, we focused our research on phosphorylated ERK1/2 protein and established a high-throughput system using the AlphaScreen\textsuperscript{®} SureFire\textsuperscript{®} Phospho-ERK assay. We validated the assay by time (not shown), and vehicle responses and using untransfected HEK293 cells (Figure 1 and Table 1).

LPI (0.1% DMSO) produced a maximal stimulation of 129.0% ± 9.65 (E\textsubscript{max}) (Figure 1A) which was not significantly different from the maximal stimulation with 0.2% DMSO (n=15 each in triplicate; Table 1; e.g. Figure 2A). The potency of LPI was not different either (Table 1). In untransfected HEK293 cells, no stimulation of phosphorylated ERK1/2 was detected at any concentration of LPI, in any given concentration of DMSO (Figure 1C). These results are similar to the results published by other groups who reported the potency of LPI in ERK1/2 phosphorylation assays (5,13) or other readouts (6).

LPI induces sustained activation of ERK1/2 phosphorylation in hGPR55-HEK293 cells. Sustained activation of ERK1/2
phosphorylation has been implicated as a measure for cancer progression, increase in cell metastasis and invasiveness of tumour cells (20,21). Importantly, GPR55-induced ERK1/2 phosphorylation regulates human cancer cell migration in vitro and proliferation in vivo (3,8,11). It also appears to govern the maintenance of persistent inflammatory pain (22,23). Therefore, we investigated the ability of LPI to maintain ERK1/2 phosphorylation after 20 min and 60 min incubation (Figure 1A). The potency of LPI after 20 min incubation, with an EC₅₀ of 0.27 µM (0.10-0.76), was significantly (p<0.05) reduced to 4.61 µM (0.75-28.3) after 60 min. Prolonged incubation reduced both basal and LPI-induced stimulation (Figure 1B, Table 1) but did not affect the net effect of LPI-induced maximal stimulation of ERK1/2 phosphorylation (Figure 1A), showing that percent stimulation was sustained. This is in contrast, for example, to CBD (10 µM) which significantly increased the percent stimulation after 60 min (Supplementary figure 2).

Structural analogues of SR141716A inhibit LPI-induced activation of ERK1/2 phosphorylation: Implications for allosteric inhibition. There is still a major controversy surrounding the profile of the arylpyrazoles at GPR55. In some studies SR141716A is reported to be an inhibitor (6) and in other studies the compound is reported to be an agonist of GPR55 (5,13,14). This is of major importance as SR141716A (Rimonabant) was widely prescribed to patients as an anti-obesity agent and was withdrawn due to mental health issues. We compared the ability of AM251, AM281, SR141716A and ABD824, an AM251 analogue in which the iodine was substituted with bromine (Figure 2, Tables 1 and see Table 2 for structures), to induce ERK1/2 phosphorylation in hGPR55-HEK293 cells with that of LPI. The efficacy of AM251 was not significantly different from that of LPI but the compound was significantly less potent (Figure 2A, Table 1). It was not possible to obtain an accurate measurement of the E₅₀ for SR141716A and AM281, however the compounds had efficacy that was not lower than that of LPI (Figure 2A and 2B; Table 1).

Interestingly, ABD824 had a bi-phasic response in hGPR55-HEK293 cells (Figure 2C).

Arylpyrazole-induced stimulation appears to be GPR55 mediated as these ligands do not stimulate ERK1/2 phosphorylation in non-expressing HEK293 cells (4) and CP55940 alone did not induce a significant stimulation of ERK1/2 phosphorylation at any concentration (Figure 2B).

In some studies SR141716A was reported to inhibit GPR55 activity (6), we therefore assessed if these arylpyrazoles could inhibit LPI-induced GPR55 stimulation, in the same experimental system. The arylpyrazoles AM251, AM281 SR141716A and ABD824 reduced the maximal stimulation (E₅₀) of LPI-induced stimulation of ERK1/2 phosphorylation but did not significantly alter the potency of this bioactive lipid (Results are summarised in Figures 2E-F and in Table 2).

Importantly, these data provide the first demonstration of a dual action (both agonist and inhibitor) of these compounds in the same assay. The inhibition appears non-competitive and may indicate allosteriq. Of the tested analogues, AM251 was the most effective as an inhibitor of LPI (Figure 2D). The E₅₀ of LPI of 147.2% ± 17.8 was reduced to 108.6% ± 3.86 by 100 nM and to 68.7% ± 9.93 (p<0.01) by 1 µM AM251. We continued to explore the effects of high concentrations of AM251 and SR141716A. The maximal stimulation of LPI in the presence of high concentrations of AM251 or SR141716A was not significantly different from that of LPI only. This would be expected because the compounds are agonists alone at these concentrations.

Previous studies reported that CP55940, a non-selective cannabinoid receptor agonist, is an inactive ligand at GPR55 that could not induce calcium response (6) but behaves as a competitive antagonist in the presence of LPI (4); another group showed it antagonised LPI, AM251 and SR141716A-induced β-arrestin trafficking and LPI-induced ERK1/2 phosphorylation (5). For comparison, in this study, LPI-induced stimulation was 144.0% ± 16.7 and CP55940 at 1 µM reduced it by only 13% (124.9% ± 16.0) which was not significantly different from LPI alone (Supplementary figure 1C).
Effect of CB$_2$ agonists on LPI-induced activation of ERK1/2 phosphorylation: Implications for neuropathic pain and positive allosteric modulation. The antinociceptive effect of CB$_2$ receptor agonists has been extensively investigated (reviewed by (24)). Subsequently, CB$_2$ receptor agonists have been developed by scientists and pharmaceutical companies as alternative treatments aimed at alleviation of neuropathic pain. Interestingly, a comparison of the behavioural responses of wild type mice with GPR55 knockout mice in pain models revealed the involvement of GPR55 in the conduction of neuropathic pain (1). These results suggest that the analgesic effects of certain CB$_2$ receptor-selective ligands may be mediated by GPR55. We compared several CB$_2$ agonists that have been intensively investigated in the past and thus form a core of active structures for GPR55-mediated ERK1/2 phosphorylation. JWH-133 (up to 3 µM) significantly reduced the basal pERK levels. GW405833 behaved as a partial agonist of GPR55 ($E_{max}$ 54% ± 13.0 vs. LPI 106.0% ± 10.5; p<0.05), with a similar potency to that of LPI (1.9 µM (0.3-10)) (Figure 3A). In contrast to the arylpyrazoles, GW405833 enhanced the LPI-induced pERK phosphorylation at a concentration which alone had no effect on pERK (Figure 3B). Conversely, JWH-133 at 1 µM and 3 µM (Figure 3C), but not at 10 µM (results not shown), inhibited the LPI-induced response. These results further support the data obtained with the arylpyrazoles, indicating that cannabinoids can act as non-competitive inhibitors of LPI signalling. Of the tested compounds, BCP ((E)-beta-caryophyllene), HU-308 (not shown) and AM1241 had little/no effect up to a concentration of 10 µM (Figure 3D) and did not significantly alter the LPI-induced response of GPR55 (Figure 3E, 3F).

Effect of phytocannabinoids on LPI-induced activation of ERK1/2 phosphorylation: Comparison of Δ$_9$-THC and Δ$_9$-THCV. Similarly to arylpyrazoles, the effect of Δ$_9$-THC, the main psychoactive constituent of Cannabis Sativa, on GPR55 is also controversial. It has been shown to increase calcium release (6) but not to mobilise β-arrestin (5) in GPR55 expressing cells. In this study, we compared the structurally related cannabis constituents, Δ$_9$-THC and Δ$_9$-THCV (Figure 4A, Table 3). Both compounds induced the stimulation of ERK1/2 phosphorylation. However, for neither compound could the stimulation be fitted on a concentration-response curve. Compared with the maximal stimulation of LPI (129.0% ± 9.65; Figure 1A), these results suggest that Δ$_9$-THC is a low affinity partial agonist while Δ$_9$-THCV is a low affinity agonist of GPR55 receptor. The levels of ERK1/2 phosphorylation with Δ$_9$-THC or Δ$_9$-THCV after prolonged incubation (60 min) were not significantly different from those obtained after 20 min.

We then assessed the modulation of LPI-induced stimulation with 1 µM Δ$_9$-THC or 1 µM Δ$_9$-THCV (produced stimulation of 22.2% ± 11.1 (not significant from zero) and 31.4% ± 10.0 (p<0.01 vs. zero), respectively). Incubation of LPI with 1 µM of Δ$_9$-THC or Δ$_9$-THCV significantly inhibited LPI-induced stimulation (Figure 4B and 4C, respectively, Table 3). Neither ligand affected the potency of LPI (Table 3). These results report for the first time that the phytocannabinoids Δ$_9$-THC and Δ$_9$-THCV are inhibitors of LPI. Moreover, Δ$_9$-THCV elicited a downward shift in the log concentration-response curve of LPI, such that basal pERK was lower in the presence of both compounds, this resembles negative cooperatively.

Structural-analogues of cannabidiol. We have recently shown that CBD antagonises LPI-induced stimulation of [35S]GTPyS binding in the breast cancer MDA-MB-231 cells, which highly express GPR55 (3). CBD also inhibits the metastasis and aggressiveness of brain and breast cancer cells (25,26) and LPI-induced calcium mobilisation in prostate cancer cells (8). Therefore, we have evaluated two cannabis constituents that are structurally related to CBD, alone or in combination with LPI. Cannabidiol acid (CBDA) has an acid group (COOH) on the benzene ring, while cannabidivarin (CBDV) has a shorter side chain compared with that of CBD (see Table 3 for structures).

We compared the effects of CBD from two sources (Tocris and GW Pharmaceuticals). The effect of CBD on ERK1/2 phosphorylation
after 20 min was not significantly different between the two sources (Supplementary figure 2A); however, after 60 min incubation, CBD significantly increased ERK1/2 phosphorylation (100.5% ± 11.9, 220.9% ± 33.2 each at 10 µM, Tocris or GW, respectively). Neither CBD nor CBDA affected basal pERK after 20 min incubation while CBDV produced a maximal stimulation of 43.0% ± 23.0 and an EC_{50} of 1.9 µM (CI, 0.13 - 27.9 µM) (Supplementary figures 2B and 2C, respectively).

The effects of CBD, CBDA and CBDV, on LPI-induced ERK1/2 phosphorylation are summarised in Table 3 and Figures 5A-5C. CBD did not significantly alter LPI efficacy or potency at 1 µM or 3 µM (Table 3). However, the inhibition of 32% at 3 µM showed a trend towards antagonism. In fact, we have found similar results using the [35S]GTPγS binding assay with MDA-MB-231 breast cancer cells (3). In these endogenously expressing GPR55 receptor cells, LPI induces the stimulation of [35S]GTPγS binding while 1 µM CBD produces a significant downward shift (6.6% ± 5.35 vs. (-)17.5 ± 3.69 with CBD) but did not affect the maximal stimulation (E_{max}) of LPI-induced [35S]GTPγS binding (LPI, 50.1% ± 7.5; 48.3% ± 21.2). However, these cells express a spectrum of receptors making them not ideal for further characterization of the pharmacology of GPR55.

GPR55 signalling involves a crosstalk between MAPK and Rho GTPases signalling pathways. We then tested if LPI-induced
stimulation of ERK1/2 phosphorylation can be inhibited by the MEK1/2 non-competitive inhibitor PD98059. LPI (10 nM to 10 µM) did not stimulate the phosphorylation of ERK1/2 proteins in the presence of PD98059, which significantly inhibited basal pERK alone (Figure 6A, 6B). These findings were in line with the independent-receptor mechanism of action of PD98059 by binding the inactive form of MEK1 and inhibiting its activation by upstream activators such as Raf kinases. However, this could not explain previous observations showing that GPR55 is predominantly coupled to Gα12/13. To test our hypothesis for crosstalk between MAPK and Rho GTPases signalling pathways, we co-incubated LPI with Y27632, a p160ROCK (Rho-associated kinase) inhibitor. After 20 min, 10 µM Y27632 significantly inhibited LPI-induced stimulation of ERK1/2 phosphorylation (Figure 6A; p<0.01), without a significant change in LPI potency. Although 10 µM Y27632 or 10 µM LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K), could equally stimulate ERK1/2 phosphorylation (Figure 6B), only Y27632 significantly inhibited the response to LPI under these conditions.

Enhancement of GPR55 signalling by PLX-4720 oncogene B-Raf inhibitor. B-Raf is a member of Raf kinase family and plays a major role in regulating ERK1/2 phosphorylation. Importantly, inherited and acquired mutations, such as V600E, in B-Raf are associated with various diseases of which most are cancers (27). Therefore, B-Raf inhibitors have been developed as anti-cancer therapeutics. PLX-4720, a potent and selective B-Raf inhibitor (28), induced a concentration-dependent stimulation of ERK1/2 phosphorylation at a magnitude similar to that induced by LPI (Figure 6C), opposing the inhibitory effects of the MEK signalling pathway in BRAF(V600E) expressing cells. In the presence of LPI, increasing concentrations of PLX-4720 (100, 300, 1000 nM) significantly increased the E\text{max} of LPI (119.2% ± 10.53) reaching 220.6% ± 13.97 at 1000 nM (p<0.01, one-way ANOVA) but did not alter its potency, this suggests an additive relation between intracellular inhibition of B-Raf and extracellular stimulation of LPI-induced of ERK1/2 phosphorylation, supporting a cross-talk between MAPK and Rho GTPases signalling pathways.

DISCUSSION
The lack of appropriate radiolabelled ligands for GPR55 precludes the characterisation of the binding of novel small molecules to this receptor. In this study, we established a new rapid and sensitive AlphaScreen® SureFire® assay as a strategy to study the pharmacology of GPR55. This method has been used to explore ligands of the cytokine receptors (29), LDL endothelial receptor (30), potassium channel TREK-1 receptors (31) and to develop antagonists for other intracellular targets (32-34).

LPI and certain cannabinoids induce sustained GPR55-mediated ERK1/2 phosphorylation
Studies have shown that GPR55 induces maximal ERK1/2 phosphorylation response after 10-20 min ((4,5,7) and this study); here we show that, although that both basal and LPI-induced stimulation levels are reduced at 60 min (Figure 1 compared with 20 min), the percent stimulation is sustained but the potency of LPI is significantly decreased after 60 min.

Sustained ERK1/2 activation in injured neurones has been suggested to reflect alterations in the intracellular feedback regulators that normally function to terminate signalling responses (35) and has been associated with brain ischemia (36) ERK1/2 phosphorylation is also associated with a variety of human pathologies (37). In addition, many mutations of components upstream to ERK1/2 alter this signalling pathway and have been associated with increased cancer metastasis and invasiveness (20,37). The combination of strength and duration of ERK1/2 signalling determines the distinct outcomes, ranging from sustained high activation that can lead to apoptosis or differentiation, to sustained lower levels of activation that are correlated with cell proliferation (38). In our case, it appears that LPI-induced activation parallels the latter scenario, supporting the role of GPR55 in cancers. Therefore, it will be interesting to determine if this mechanism involves the
endogenous mitogen-activated protein kinase phosphatase 1 (MKP-1) that controls the constitutive activation of ERK1/2 (21).

**GPR55 pharmacology of arylpyrazoles**

A key finding of this study is the demonstration that arylpyrazoles, e.g. SR141716A, can act both as agonists of GPR55 and inhibitors of LPI-induced activation of pERK. Thus here we demonstrate for the first time, that both types of behaviour (agonism and inhibition) that had been reported by previous studies from different laboratories can exist in the same model system. Our data suggest that there may be two, topographically distinct, binding sites on GPR55 (Figure 7): in one scenario, certain ligands (e.g. AM251, GW405833) could act as agonists alone (either via the orthosteric (LPI) binding site or a distinct (allosteric) binding site; (e.g. (39)). In second scenario the same ligands could act as modulators of LPI-induced pERK activation. This suggests that the compounds may bind to an allosteric site to induce a conformational change in the orthosteric (LPI) binding site such that the efficacy of LPI is reduced (e.g. AM251) or enhanced (e.g. GW405833). These observations raise the possibility that certain arylpyrazole ligands may represent bitopic ligands of GPR55. Bitopic ligands have the capacity to interact with both the orthosteric site (as agonists) and the allosteric site (as modulators) through distinct chemical structures (40).

Allosteric binding sites have been described for other lipid receptors, including allosteric modulators of anandamide at the cannabinoid CB1 receptor (41) and ago-allosteric ligands for short-chain free fatty acids at FFA2 and FFA3 receptors (42). The current study demonstrates the modulation of GPR55 by certain ligands, possibly by allosteric mechanism. The first evidence is the reduction in $E_{\text{max}}$ of LPI observed in the presence of various compounds. However, it is important to note that this study does not provide direct evidence of allostery (e.g. dissociation kinetics) and there is a possibility that the compounds may inhibit LPI signalling downstream of the receptor to disrupt the pERK signalling cascade.

The second evidence is the apparent negative cooperatively between various ligands and LPI such that the basal levels of pERK are significantly decreased in the presence of both ligands; an effect not observed with either ligand alone.

Clearly, this evidence is circumstantial and dissociation kinetic analysis, which is not currently feasible due to the lack of appropriate radioligand, or extensive mutation studies, will be required to confirm the existence of topographically distinct binding sites at GPR55. Nevertheless, this study provides the first potential explanation for the considerable controversy in the pharmacology of cannabinoid ligands at GPR55 and has important therapeutic implications.

The arylpyrazoles belong to a subgroup of ligands that interact with both GPR55 and CB1 receptors. In this study, arylpyrazole analogues induced activation of pERK with a rank order of efficacy LPI >> AM251 > SR141716A >>> AM281. This rank of potency is in agreement with the Henstridge et al. study (2009) using the same hGPR55-expressing HEK293 cells and is similar to the Kapur et al. study (2009) (5) which used hGPR55-expressing U2OS cells and a β-arrestin mobilisation assay. Although in the latter study arylpyrazoles could not induce the phosphorylation of ERK1/2, the difference between this and our study may be due to differences in method sensitivity (Western blot vs. AlphaScreen®) or may reflect cell type.

Collectively, these results suggest that AM251, SR141716A and AM281 are weak agonists of GPR55 as measured by their ability to induce ERK1/2 activation. Interestingly ABD824, an AM251 analogue, decreased basal pERK at concentrations below 3 µM. This suggests a role for the chemical group on the aryl ring in shifting the GPR55 receptor between inactive and active receptor conformations. As for the efficacy for the observed antagonism of LPI by arylpyrazoles, the rank order is AM251 > AM281 > ABD824 ≥ SR141716A which does not correspond to the potency of these ligands as GPR55 agonists (as detailed above). This suggests that the pharmacophore for inhibition of GPR55 by these pyrazoles is different to that responsible for activation of GPR55.
SR141716A was developed as a cannabinoid CB\textsubscript{1} receptor antagonist and progressed to clinical studies as a drug treatment for obesity related disorders (43). Interestingly, SR141716A has been shown to reduce neuropathy associated with type-2 diabetes in patients (44). This has also been supported by in \textit{vivo} studies in which SR141716A reduced neuropathic pain in a murine model for obesity (45) and in a rodent model for nerve injury (46). This is unexpected from an inverse agonist of CB\textsubscript{1} because CB\textsubscript{1} receptor agonists are effective in neuropathic pain. Collectively, these studies suggest that SR141716A may act on a target other than the CB\textsubscript{1} receptor to inhibit pain. As GPR55 knock-out mice are less sensitive to the development of chronic pain (1), our findings that LPI-induced activation of GPR55 is inhibited by SR141716A suggests a novel mechanism of action for the regulation of pain and possibly for metabolic syndrome by this compound. This effect may be mediated by a putative allosteric binding site on GPR55.

\textbf{GPR55 pharmacology of CB\textsubscript{2} agonists}

This study has identified GW405833 as a GPR55 partial agonist. JWH-133 significantly reduced basal pERK and as such may be an inverse agonist of GPR55. In line with our results, JWH-133 has been previously studied for its analgesic effects and has shown to produce antinociception via nicotinic-dependent pathways (47). However, its effect could not always be reversed by SR144528, a CB\textsubscript{2} receptor antagonist (48). Similarly, GW405833 was found to promote a pro-nociception response in a rat model for osteoarthritis in contrast to its behaviour as partial agonist of CB\textsubscript{2} receptor (49). These studies suggest that JWH-133 and GW405833 have an additional target/s and our results suggest that GPR55 signalling may be one of their targets.

\textit{Phytocannabinoids that inhibit LPI-mediated activation of GPR55}

The pharmacological actions of several \textit{Cannabis Sativa} constituents have been recently reviewed; both the psychoactive and non-psychoactive compounds have multiple targets and convey a range of pharmacological actions (50). In this study we have evaluated a range of \textit{Cannabis Sativa} constituents for their ability to modulate GPR55. While \Delta^9-THC activates CB\textsubscript{1} and CB\textsubscript{2} receptors and GPR55, and behaves as a partial agonist at these receptors, \Delta^9-THCV is a CB\textsubscript{1} antagonist and a CB\textsubscript{2} partial agonist. In this study we found that \Delta^9-THCV is a weak agonist of GPR55 that can significantly inhibit LPI-induced stimulation of pERK in GPR55 expressing cells. Thus, the analgesic actions of \Delta^9-THCV \textit{in vivo} (51) might, at least in part, be mediated by modulation of GPR55. Expression of GPR55 mRNA has been detected in adipose tissue (15) and a polymorphism in the GPR55 gene has been found in females with anorexia nervosa (52). Whilst the precise role of GPR55 in food intake and metabolism remains to be elucidated, it is possible that the inhibition of food intake reported for \Delta^9-THCV (53) and other cannabinoids may be mediated, at least in part, by modulation of GPR55.

Here we report that the little investigated cannabis constituents, CBDV, CBGA and CBGV are potent inhibitors of LPI-induced GPR55 signalling. CBD is the most investigated non-psychotropic constituent of cannabis and has a wide range of pharmacological targets (50); together with a safe profile in humans, this made it highly attractive for the development of drugs for neuropathic pain associated with multiple sclerosis (54) and cancer (55). Several studies have also shown that CBD is effective against proliferation, migration and invasion of a variety of breast cancer, glioblastoma, prostate cancer and human cervical cancer cell lines (3,8,25,26,56). In cancer cells, the target for CBD remains elusive while the actions of \Delta^9-THC have so far been considered to be mediated by activation of cannabinoid receptors. Recently has GPR55 been linked to inhibition of the migration and proliferation of cancer cells by CBD (3,8). The results warrant further investigation into the potential therapeutic use of various phytocannabinoid. CBDA and CBG have been shown to activate TRPA1 and TRPV1-expressing cells and to antagonise TRPM8, implying a role in analgesia and prostate cancer for these compounds (57,58). Our study suggests that GPR55 is an additional target for these compounds; inhibition of GPR55...
would therefore further support their potential role in the treatment of carcinoma and pain (50).

GPR55- mediated MAP kinase signalling

Importantly, our findings demonstrate that LPI-induced ERK1/2 phosphorylation is controlled, at least in part, by ROCK, supporting the role for Go_{12/13} in LPI signalling and indicating crosstalk between MAPK and Rho GTPases signalling and in line with Andradas et al. (2011) (11). Such crosstalk may be communicated via the serine/threonine kinase Raf family, of which the expression level of the oncogene B-Raf in turn controls the stimulation of ROCK (59). In line with Hatzivassiliou et al., (2010) (16), the selective B-Raf inhibitor, PLX-4720 unregulated ERK1/2 phosphorylation in hGPR55-HEK293 cells which do not carry the BRAF(V600E) mutation, interestingly, the magnitude of the response closely resembled that of LPI. Furthermore, ERK1/2 phosphorylation was unregulated following the co-incubation of PLX-4270 with LPI in an additive manner, supporting a cross talk between B-Raf and ROCK signalling pathways in LPI-mediated GPR55 signalling. Further support for this interaction comes from B-Raf knockout mice in which ROCKII expression is reduced (60).

Conclusions

We have established a rapid and sensitive method to study the pharmacology of the GPCR GPR55 using the AlphaScreen® SureFire® ERK assay. It is important to note that this study exclusively measures ERK1/2 phosphorylation as readout of GPR55. This readout may not allow the distinction within various signalling pathways, thus, this work might be limited by the fact that the signalling cascade leading to ERK1/2 activation may be variable depending the compound used. That said, to our knowledge this study provides the first evidence that certain cannabinoids can display both activation of GPR55 and inhibition of LPI-mediated pERK stimulation in the same assay. This may go some way to resolving the controversy regarding the pharmacology of cannabinoids at GPR55. Furthermore, we show that certain ligands previously thought to be selective for CB2 receptor, also modulate GPR55. Our findings also suggest that GPR55 may be a new pharmacological target for the following Cannabis Sativa constituents: Δ^9-THCV, CBDV, CBGA and CBGV. Combined mutagenesis and pharmacological investigations will enable us to determine the pharmacophores responsible for cannabinoid binding to GPR55 to facilitate rational drug design (61). This study has implications for developing new therapeutics for the treatment of cancer, pain and metabolic disorders.
REFERENCES


This work was supported by the US National Institutes of Health grant numbers DA-03672 and DA-09789 (RAR, RGP). We thank University of Aberdeen for a Knowledge Transfer Grant Award.

Conflicts of interest: RGP and RAR received funding from GW Pharmaceuticals.

Abbreviations: AM281, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide; AM251, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; CBD, Cannabidiol; CBDA, Cannabidiol acid; CBDV, Cannabidivarin; CBG, Cannabigerol; CBGA, Cannabigerolic acid; CBGV, Cannabigerovarin; ERK1/2, extracellular signal-regulated kinases 1/2; GPR55, G protein-coupled receptor 55, GPR55-HEK293, stable cell line expressing 3xHA-GPR55; HA, hemagglutinin; LPI, L-α-lysophosphatidylinositol; SR141716A, 5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-N-(piperidin-1-yl)-1H-pyrazole-3-carboxamide; Δ9-THC, Delta(9)-Tetrahydrocannabinol; Δ9-THCV, Delta(9)-Tetrahydrocannabivarin.

FIGURE LEGENDS

Figure 1. Detection of LPI-induced ERK1/2 phosphorylation in hGPR55-HEK293 cells using the AlphaScreen® SureFire® ERK assay. (A) Mean log concentration-response curves of LPI effect on ERK1/2 phosphorylation in hGPR55-HEK293 cells after 20 min (n=4 each in triplicate) or 60 min (n=3 each 2-3 repeats) incubation at 37°C. Increasing the incubation time significantly (p<0.05) reduced the potency of LPI-induced ERK1/2 phosphorylation but did not affect LPI-induced maximal stimulation (Eₘₐₓ) of GPR55 receptor. (B) Compared with 20 min incubation time at 37°C, 60 min incubation significantly (p<0.05) reduced the basal level of ERK1/2 phosphorylation (n=4, each in 12 repeats). Raw data of AlphaScreen® SureFire® ERK assay are presented as ‘Envision units’, Student t-test,*p<0.05. (C) No significant differences were observed in basal levels of phosphorylated ERK1/2 in untransfected HEK293 and hGPR55-HEK293 incubated for 20 min in 0.1% or 0.5 % DMSO. (C) The effect of DMSO on LPI-induced ERK1/2 phosphorylation was assessed at 0.1% or 0.5% DMSO in either hGPR55-HEK293 cells or untransfected HEK293 cells. In untransfected HEK293 cells, no stimulation of ERK1/2 phosphorylation was detected at any concentration of LPI at any concentration of DMSO (untransfected and hGPR55-HEK293 cells were seeded on the same plate). Each symbol represents the mean percentage change in bound phosphorylated ERK1/2 protein.

Figure 2. Structural analogues of SR141716A induced ERK1/2 phosphorylation in hGPR55-HEK293 cells. (A) Mean log concentration-response curves of percent stimulation of ERK1/2 phosphorylation by LPI (n=4), AM251 (n=3) or AM281 (n=3) after 20 min stimulation at 37°C. (B) Mean log concentration-response curves of LPI or ABD824, an AM251 analogue in which iodine was substituted with a bromine (n=3, each in duplicate). The effect of SR141716A analogues on LPI-induced ERK1/2 phosphorylation in hGPR55-HEK293 cells. (D) with LPI (n=4) in the presence or absence of 100 nM AM251 (n=3) or 1 µM AM251 (n=4). (E) The effect of LPI in the presence or absence of 1 µM SR141716A (n=4) or 1 µM AM281 (n=3). (F) Effect of LPI in the presence or absence of 1 µM and 3 µM ABD824 (n=4, each in duplicate). Each symbol represents the mean percentage change in bound phosphorylated ERK1/2 ± SEM over the basal level, each independent experiment was performed in triplicate unless stated otherwise.
Figure 3. The effect of CB2 receptor agonists on ERK1/2 phosphorylation in hGPR55-HEK293 cells. (A) Mean log concentration-response curves of percent stimulation of ERK1/2 phosphorylation by LPI (n=7), GW405833 (n=4) or JWH-133 (n=3) after 20 min stimulation at 37°C. JWH-133 significantly reduced basal pERK levels, *p<0.05, **p<0.01, one-sample t-test. (B) GW405833 at 10, 30 and 100 nM enhanced the LPI-induced ERK1/2 phosphorylation (n=4) (C) JWH-133 at 1 and 3 μM inhibited the LPI-induced ERK1/2 phosphorylation (n=4) (D) Percent stimulation of ERK1/2 phosphorylation by LPI (n=3), AM1241 (n=4) or HU-308 (n=4) and BCP (n=3) after 20 min stimulation at 37°C. (E) BCP at 1, 3 and 10 μM (n=3) did not alter the LPI-induced ERK1/2 phosphorylation nor did AM1241 (F, n=3). Each symbol represents the mean percentage change in bound phosphorylated ERK1/2 ± SEM over the basal level, each independent experiment was performed in duplicate.

Figure 4. Effect of Δ9-THC and Δ9-THCV on ERK1/2 phosphorylation in hGPR55-HEK293 cells. Mean log concentration-response curves of ERK1/2 phosphorylation after 20 min or 60 min stimulation at 37°C with (A) Δ9-THC (n=3) or Δ9-THCV (n=4). (B) Shows the effect of LPI in the presence or absence of 1 μM Δ9-THC after 20 min stimulation at 37°C (n=4). (C) Shows the effect of LPI in the presence or absence of 1 μM Δ9-THCV after 20 min stimulation at 37°C (n=3). Each symbol represents the mean percentage change in bound phosphorylated ERK1/2 ± SEM over the basal level, each independent experiment was performed in triplicate.

Figure 5. Effect of CBD and CBDA and CBDV on ERK1/2 phosphorylation in hGPR55-HEK293 cells. Mean log concentration-response curves of ERK1/2 phosphorylation after 20 min stimulation at 37°C, the effect of LPI in the presence or absence of (A) 1 μM CBD (n=5), (B) 1 μM CBDA (n=3) or (C) 1 μM CBDV (n=3), (D) 1 μM CBG (n=4), (E) 1 μM CBGA (n=4) or (F) 1 μM CBGV (n=3). CBDV and CGV pronouncedly inhibited LPI compared with their analogues. Each symbol represents the mean percentage change in bound phosphorylated ERK1/2 ± SEM over the basal level (n=3). CBDA, CBDV, CBGV significantly reduced basal pERK levels, *p<0.05, **p<0.01, ***p<0.001 one-sample t-test.

Figure 6. The effect of kinase inhibitors. (A) LPI induced-stimulation of ERK1/2 phosphorylation was attenuated by 10 μM PD98059, a MEK1 inhibitor and inhibited by 10 μM Y27632, a Rho-associated protein kinase inhibitor (n=5 each in duplicate) but not by 10 μM LY294002 a phosphatidylinositol 3-kinase inhibitor (n=3 each in duplicate). (B) The effect of each inhibitor alone on phosphorylated ERK1/2 level (n=4 for PD98059; n=7 for Y27632; n=3 for LY294002, each in duplicate). PD98059 significantly reduced basal pERK levels, *p<0.05, **p<0.01, ***p<0.001 one-sample t-test. (C) Diagram representing the putative GPR55/LPI signalling cascade (D) The selective B-Raf inhibitor, PLX-4720, stimulated hGPR55-HEK293 expressing cells in a similar manner to this of LPI (n=3 each in duplicate). (E) Increasing concentrations of PLX-4720 significantly increased the efficacy of LPI-induced response (100 nM, p< 0.05; 300 nM, p<0.01; 1000 nM, p<0.01) and the bottom of the curve (300 nM, p<0.05), (n=3 each in duplicate).

Figure 7. Pharmacological mechanisms for the modulation of GPR55. LPI is suggested to primarily bind the GPR55 orthosteric binding site. GPR55 may also contain an allosteric binding site. These observations raise at least two possibilities: (A) One is that certain arylpyrazole ligands actually represent bitopic ligands of GPR55. These ligands may have the capacity to modulate both the orthosteric (agonists) and the allosteric site through distinct chemical structures. Second is that AM251 and certain arylpyrazole analogues are only allosteric ligands that are ago-allosteric alone. (B) In the presence of LPI, arylpyrazoles can also behave as allosteric inhibitors and GW405833 as allosteric enhancer. In addition, a number of Cannabis Sativa constituents appear to inhibit ERK1/2 phosphorylation in an allosteric manner.
<table>
<thead>
<tr>
<th>Compound</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (95% CL) (µM)</th>
<th>% E&lt;sub&gt;max&lt;/sub&gt; (SEM)</th>
</tr>
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<tbody>
<tr>
<td>LPI (0.1% DMSO) 20 min</td>
<td>0.27 (0.10-0.76)</td>
<td>129.0 ± 9.65</td>
</tr>
<tr>
<td>LPI (0.2% DMSO) 20 min</td>
<td>1.12 (0.59-2.16)</td>
<td>126.0 ± 9.44</td>
</tr>
<tr>
<td>LPI (0.1% DMSO) 60 min</td>
<td>4.61 (0.75-28.26) *</td>
<td>139.8 ± 45.70</td>
</tr>
<tr>
<td>AM251</td>
<td>2.34 (0.99-5.56)**</td>
<td>153.2 ± 22.10</td>
</tr>
<tr>
<td>ABD824</td>
<td>0.7 (0.11-4.44)</td>
<td>(-)34.16 ± 22.4 (up to 1 µM)</td>
</tr>
<tr>
<td>AM281</td>
<td>NA</td>
<td>~ 106 (10 µM)</td>
</tr>
<tr>
<td>SR141716A</td>
<td>NA</td>
<td>~ 160 (10 µM)</td>
</tr>
<tr>
<td>CP55940</td>
<td>NA</td>
<td>~ 50 (10 µM)</td>
</tr>
<tr>
<td>JWH-133</td>
<td>0.16 ((-)30.98 – (-)4.41)</td>
<td>(-)29.8 ± 6.40 (up to 3 µM)</td>
</tr>
<tr>
<td>GW405833</td>
<td>1.87 (0.33 – 10.7)</td>
<td>54.0 ± 13.04</td>
</tr>
<tr>
<td>HU-308</td>
<td>NA</td>
<td>~ 20 (10 µM)</td>
</tr>
<tr>
<td>AM1241</td>
<td>NA</td>
<td>~ 15 (10 µM)</td>
</tr>
<tr>
<td>BCP</td>
<td>NA</td>
<td>~ 22 (10 µM)</td>
</tr>
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</table>

Cells were treated with each drug for 20 min or for the indicate time at 37°C. *P<0.05, **P<0.01, vs. LPI in 0.1% DMSO for 20min (first raw).
<table>
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<tr>
<th>Arylpyrazole</th>
<th>LPI ± drug</th>
<th>EC(_50) (µM)</th>
<th>pEC(_{50}) ± SEM</th>
<th>% E(_{\text{max}}) ± SEM</th>
<th>% Inhibition</th>
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<td><img src="image1" alt="Arylpyrazole" /></td>
<td>Vehicle</td>
<td>1.15</td>
<td>5.94 ± 0.22</td>
<td>147.2 ± 17.76</td>
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<td>AM251 100 nM</td>
<td>0.53</td>
<td>6.28 ± 0.07</td>
<td>108.6 ± 3.86</td>
<td>26%</td>
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<td>AM251 1 µM</td>
<td>0.67</td>
<td>6.17 ± 0.29</td>
<td>68.70 ± 9.33 **</td>
<td>53%</td>
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<td><img src="image2" alt="Arylpyrazole" /></td>
<td>Vehicle</td>
<td>0.79</td>
<td>6.10 ± 0.20</td>
<td>161.9 ± 12.14</td>
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<tr>
<td></td>
<td>SR141716A 1 µM</td>
<td>0.56</td>
<td>6.25 ± 0.29</td>
<td>108.3 ± 14.3*</td>
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<tr>
<td><img src="image3" alt="Arylpyrazole" /></td>
<td>Vehicle</td>
<td>0.51</td>
<td>6.29 ± 0.24</td>
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<td>ABD824 1 µM</td>
<td>0.45</td>
<td>6.35 ± 0.37</td>
<td>70.39 ± 10.65</td>
<td>34%</td>
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<tr>
<td></td>
<td>ABD824 3 µM</td>
<td>0.42</td>
<td>6.38 ± 0.58</td>
<td>59.7 ± 11.7*</td>
<td>44%</td>
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<tr>
<td><img src="image4" alt="Arylpyrazole" /></td>
<td>Vehicle</td>
<td>0.89</td>
<td>6.05 ± 0.12</td>
<td>131.6 ± 8.9</td>
<td></td>
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<tr>
<td></td>
<td>AM281 1 µM</td>
<td>0.25</td>
<td>6.59 ± 0.24</td>
<td>81.49 ± 8.71 *</td>
<td>45%</td>
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Cells were co-treated with LPI in the presence or absence of a given drug at 1 µM for 20 min at 37°C. Final concentration was 0.2% DMSO. *P<0.05, ** P<0.01 vs. LPI in each experiment. % Inhibition is percentage inhibition of E\(_{\text{max}}\) relatively to LPI (100%).
<table>
<thead>
<tr>
<th>Phytocannabinoid</th>
<th>LPI ± vh or drug</th>
<th>EC$_{50}$ (µM)</th>
<th>pEC$_{50}$ ± SEM</th>
<th>% $E_{\text{max}}$ ± SEM</th>
<th>% Inhibition</th>
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<tr>
<td></td>
<td>Vehicle</td>
<td>0.80</td>
<td>6.10 ± 0.20</td>
<td>122.1 ± 11.6</td>
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<tr>
<td>$\Delta^9$-THC</td>
<td>1 µM</td>
<td>0.64</td>
<td>6.20 ± 0.18</td>
<td>87.9 ± 8.3 *</td>
<td>28%</td>
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<tr>
<td>$\Delta^9$-THCV</td>
<td>1 µM</td>
<td>0.88</td>
<td>6.06 ± 0.22</td>
<td>60.8 ± 11.3 *</td>
<td>50%</td>
</tr>
<tr>
<td>CBD</td>
<td>1 µM</td>
<td>1.03</td>
<td>5.98 ± 0.16</td>
<td>100.4 ± 10.40</td>
<td>9%</td>
</tr>
<tr>
<td></td>
<td>3 µM</td>
<td>1.68</td>
<td>5.78 ± 0.15</td>
<td>72.8% ± 9.05</td>
<td>32%</td>
</tr>
<tr>
<td>CBDA</td>
<td>1 µM</td>
<td>1.36</td>
<td>5.86 ± 0.16</td>
<td>116.6 ± 15.20</td>
<td>16%</td>
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<tr>
<td>CBDV</td>
<td>1 µM</td>
<td>0.40</td>
<td>6.40 ± 0.21</td>
<td>49.11 ± 8.80 *</td>
<td>56%</td>
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Cells were co-treated with LPI in the presence or absence of a given drug at 1 µM for 20 min at 37°C. Final concentration was 0.2% DMSO.

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

*P<0.05 vs. LPI in each experiment. % Inhibition is percentage inhibition of E\textsubscript{max} relative to LPI (100%). # 1µM unless stated otherwise.

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<tr>
<th>Phytocannabinoid</th>
<th>LPI ± 1 µM drug#</th>
<th>EC\textsubscript{50} (µM)</th>
<th>pEC\textsubscript{50} ± SEM</th>
<th>% E\textsubscript{max} ± SEM</th>
<th>% Inhibition</th>
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<tbody>
<tr>
<td><img src="image1" alt="Phytocannabinoid 1" /> Vehicle</td>
<td>0.89</td>
<td>6.05 ± 0.19</td>
<td>115.7 ± 13.7</td>
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<tr>
<td><img src="image2" alt="Phytocannabinoid 2" /> CBG</td>
<td>2.16</td>
<td>5.66 ± 0.27</td>
<td>96.7 ± 21.67</td>
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<tr>
<td><img src="image3" alt="Phytocannabinoid 3" /> Vehicle</td>
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<td>6.05 ± 0.19</td>
<td>115.7 ± 13.70</td>
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<tr>
<td><img src="image4" alt="Phytocannabinoid 4" /> CBGA</td>
<td>0.48</td>
<td>6.32 ± 0.46</td>
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<td>56%</td>
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<tr>
<td><img src="image5" alt="Phytocannabinoid 5" /> Vehicle</td>
<td>2.28</td>
<td>5.64 ± 0.18</td>
<td>133.6 ± 16.01</td>
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<tr>
<td><img src="image6" alt="Phytocannabinoid 6" /> CBGV</td>
<td>0.72</td>
<td>6.14 ± 0.26</td>
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Table 3 (continued)

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<th>CB2 receptor compound</th>
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<th>pEC50 ± SEM</th>
<th>% Emax ± SEM</th>
<th>% Inhibition</th>
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<td>JWH-133 1 µM</td>
<td>0.90</td>
<td>6.04 ± 0.12</td>
<td>80.1 ± 5.11</td>
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<tr>
<td>JWH-133 3 µM</td>
<td>0.88</td>
<td>6.06 ± 0.33</td>
<td>59.5 ± 4.16*</td>
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<table>
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<th>pEC50 ± SEM</th>
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<th>% Enhancement</th>
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<td>Vehicle</td>
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<td>6.20 ± 0.18</td>
<td>98.9 ± 9.28</td>
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<td>GW405833 10 nM</td>
<td>1.07</td>
<td>5.97 ± 0.20</td>
<td>138.0 ± 6.54</td>
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<tr>
<td>GW405833 30 nM</td>
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<td>5.73 ± 0.19</td>
<td>201.8 ± 23.97</td>
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<tr>
<td>GW405833 100 nM</td>
<td>0.35</td>
<td>6.46 ± 0.26</td>
<td>178.1 ± 18.61</td>
<td>80%</td>
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Cells were co-treated with LPI in the presence or absence of a given drug at 1 µM for 20 min at 37°C. Final concentration was 0.2% DMSO. *P<0.05, **P<0.01 vs. LPI in each experiment. % Inhibition is percentage inhibition of Emax relative to LPI (100%).
Figure 1

A

% Stimulation (pERK)

LPI (log M)

-9 -8 -7 -6 -5 -4

0.1% DMSO
0.5% DMSO

hGPR55-HEK293

B

% Stimulation (pERK)

LPI (log M)

-9 -8 -7 -6 -5 -4

0.1% DMSO
0.5% DMSO

Untransfected HEK293

C

Untransfected HEK293

hGPR55-HEK293

-8 -7 -6 -5

0.1% DMSO
0.5% DMSO

-9 -8 -7 -6 -5 -4
Figure 2

A  
\[ \text{Vehicle} \]  
\[ 1 \mu M \text{ AM251} \]  
\[ 1 \mu M \text{ AM281} \]  
\[ \text{Drug (log M)} \]  
\[ \% \text{ Stimulation (pERK)} \]  

B  
\[ \text{LPI} \]  
\[ \text{SR141716A} \]  
\[ \text{CP55940} \]  
\[ \text{Drug (log M)} \]  
\[ \% \text{ Stimulation (pERK)} \]  

C  
\[ \text{LPI} \]  
\[ \text{ABD824} \]  
\[ \text{Drug (log M)} \]  
\[ \% \text{ Stimulation (pERK)} \]  

D  
\[ \text{Vehicle} \]  
\[ 100 \text{ nM AM251} \]  
\[ 1 \mu M \text{ AM251} \]  
\[ \text{LPI (log M)} \]  
\[ \% \text{ Stimulation (pERK)} \]  

E  
\[ \text{Vehicle} \]  
\[ 1 \mu M \text{ SR141716A} \]  
\[ 1 \mu M \text{ AM281} \]  
\[ \text{LPI (log M)} \]  
\[ \% \text{ Stimulation (pERK)} \]  

F  
\[ \text{Vehicle} \]  
\[ 1 \mu M \text{ ABD824} \]  
\[ 3 \mu M \text{ ABD824} \]  
\[ \text{LPI (log M)} \]  
\[ \% \text{ Stimulation (pERK)} \]
Figure 4
Figure 7