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Title: **INHIBITION OF HUMAN NEUTROPHIL CHEMOTAXIS BY ENDOGENOUS CANNABINOIDS AND PHYTOCANNABINODS: EVIDENCE FOR A SITE DISTINCT FROM CB_1 AND CB_2.**

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Running Title: Cannabinoids and Human Neutrophil Migration

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Abstract: 233

Introduction: 503

Discussion: 1616

Figures: 6

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Abbreviations and Chemical Names

Abn-CBD, Abnormal-Cannabidiol, *trans*-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol; AEA, Anandamide, *N*-Arachidonoyl ethanolamide; 2-AG, 2-Arachidonoyl Glycerol; ARA-S, *N*-Arachidonoyl L-Serine; CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; CBD, cannabidiol; FAAH, fatty acid amide hydrolase; COX, cyclo-oxygenase; CPZ, capsazepine, *N*-[2-(4-Chlorophenyl)ethyl]-1,3,4,5- tetrahydro -7,8- dihydroxy- 2H-2-benzazepine-2-carbothioamide ; DMSO, dimethylsulphoxide; ETYA, 5,8,11,14-eicosatetraynoic acid; fMLP, N-formyl-methione-leucine-phenylalanine; HETE, hydroxyl-eicosatetraenoic; LTB_4, Leukotriene B_4; LOX, lipoxygenase; LPI, lysophosphatidylinositol; NADA, *N*-arachidonoyl dopamine; 01602, *trans*-4-[3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-methyl-1,3-benzenediol; PEA, palmitoylethanolamide; PBS, phosphate buffered saline; PMSF, phenylmethylsulphonyl fluoride; SR141716A, *N*-piperidino-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-3-pyrazole-carboxamide; Δ^9THC, Δ^9-tetrahydrocannabinol.

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Abstract

Here we show a novel pharmacology for inhibition of human neutrophil migration by endocannabinoids, phytocannabinoids and related compounds. The endocannabinoids virodhamine and *N*-arachidonoyl dopamine are potent inhibitors of fMLP-induced migration of human neutrophils, with IC_{50} values of 0.2 and 8.80 nM respectively. The endocannabinoid, anandamide inhibits human neutrophil migration at nM concentrations in a bi-phasic manner. The phytocannabinoid, (-)-cannabidiol is a partial agonist, being ~ 40 fold more potent than (+)-cannabidiol; abnormal-cannabidiol is a full agonist. Furthermore, the abnormal-CBD analogue, O-1602 inhibits migration with an IC_{50} value of 33 nM. This reported profile of agonist efficacy and potency parallels with the pharmacology of the novel 'abnormal-cannabidiol' receptor or a related orphan GPCR, which are already known to modulate cell migration. Whilst having no effect alone, *N*-arachidonoyl L-serine attenuated inhibition of human neutrophil migration induced by anandamide, virodhamine and abnormal-CBD. Our data also suggest that there is cross-talk/negative co-operativity between the cannabinoid CB_2 receptor and this novel target: CB_2 receptor antagonists significantly enhance the inhibition observed with anandamide and virodhamine. This study reveals that certain endogenous lipids, phytocannabinoids and related ligands are potent inhibitors of human neutrophil migration and implicates a novel pharmacological target distinct from cannabinoid CB_1 and CB_2 receptors; this target is antagonised by the endogenous compound, *N*-arachidoloyl L-serine. Furthermore, our findings have implications for the potential pharmacological manipulation of elements of the endocannabinoid system for the treatment of various inflammatory conditions.

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Introduction

The endocannabinoid system comprises two known receptors (CB_1 and CB_2); a family of endogenous ligands (endocannabinoids); and specific molecular machinery for the synthesis, transport and inactivation of these ligands (Pertwee and Ross, 2002). The most studied endocannabinoids are arachidonoylethanolamide (AEA), also known as anandamide, and 2-arachidonoyl glycerol (2-AG), both of which are synthesised on demand and are rapidly hydrolysed by the enzymes, fatty acid amide hydrolase (FAAH) and monoacyl glycerol lipase (MAG lipase) respectively (Fowler et al, 2005). There are a number of additional endocannabinoids; these include *N*-arachidonoyl dopamine (NADA) and virodhamine (De Petrocellis et al, 2004). The major constituents of cannabis include Δ^9-tetrahydrocannabinol (Δ^9THC), which is psychoactive and (-)-cannabidiol (CBD), which is non-psychoactive (Di Marzo and De Petrocellis, 2006).

The pharmacology of both the endocannabinoids and the phytocannabinoids appears to be increasingly complex, their actions being mediated by cannabinoid CB_1 and CB_2 receptors and by putative non-CB_1, non-CB_2 receptors; (Pertwee, 2004; Begg et al, 2005; Mackie and Stella, 2006). One such novel target, the 'abnormal-cannabidiol' (abn-CBD) receptor, has been implicated in modulation of endothelial, microglial and glioma cell migration (Mo et al, 2004; Vaccani et al, 2005; Walter et al, 2003; Franklin and Stella, 2003). Furthermore, *N*-arachidonoyl-L-serine, an endocannabinoid-like brain constituent, has been reported to be an agonist at this receptor (Millman et al, 2005). Recently it has emerged that various cannabinoids including abnormal-CBD, it's analogue O-1602, virodhamine and anandamide bind to and activate the orphan GPCR, GPR55 which is reported to be coupled to Gα13 and to activate rhoA, cdc42 and rac1 (Johns et al, 2007; Ryeberg et al, 2007; for commentaries see Hiley & Kaup, 2007 and Pertwee, 2007). In contrast, others find that lysophosphatidylinositol (LPI), but not cannabinoid ligands, induce ERK phosphorylation in GPR55-expressing cells (Oka et al, 2007).

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There is a growing body of evidence suggesting that neutrophils make a crucial contribution to a number of autoimmune, autoinflammatory and neoplastic disorders (Nathan, 2006). This study was directed at investigation of the modulation of human neutrophil migration. Cannabinoid CB_2 receptors are primarily expressed in immune cells. In neutrophils, the CB_2 receptor plays a key role in differentiation (Dercq et al, 2000; Jorda et al, 2004) and has been implicated in the development of leukaemia. 2-AG is reported to exert CB_2-mediated stimulation of hematopoietic cell migration (Walter et al, 2003; Oka et al, 2004; Jorda et al, 2002; Kishimoto et al; Kishimoto et al, 2005; Sacerdote et al, 2005). On the other hand, inhibition of migration by 2-AG, AEA, synthetic cannabinoids and the phytocannabinod, Δ^9THC, has also been reported (Sacredote et al, 2000; Steffens et al, 2005; Kurihara et al, 2006; Ghosh et al, 2006).

In this milieu, the primary aim of our study was to investigate the modulation of human neutrophil migration by cannabinoids, focussing on the underlying receptor pharmacology and the contribution, if any, of non-CB_1, non-CB_2 receptors. Included in the study are endocannabinoids and related endogenous lipids and phytocannabinoids and structurally-related ligands; specifically those that have been demonstrated to interact with either the abnormal-CBD receptor or GPR55.

Materials and Methods

Isolation of neutrophils

Polymorphprep™ is a ready made, sterile and endotoxin-tested solution designed to isolate polymorphonuclear granulocytes. Whole blood separates into distinct bands of plasma, mononuclear leukocytes, polymorphonuclear neutrophils (PMN), and red blood cells when centrifuged over the solution. 5 ml of normal whole blood was carefully layered over 5 ml of Polymorphprep™ in 12 ml centrifuge tubes. The filled tubes were centrifuged at 550 g for 35 min at 20°C and the PMN layer was removed with a fine-tipped Pasteur pipette. In order to remove the residual Polymorphprep™, the cells were suspended in a universal container with 20 ml of sterile RPMI at 37°C and centrifuged (Mistral 1000 centrifuge) at 450 g for 10 min at 20°C. The supernatant fluid was discarded and the pellet re-suspended with 10 ml of PBS

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(containing $CaCl_2$ and $MgCl_2$) and 10 ml of 4°C distilled H_2O for further washing, and centrifuged again at 450 g for 10 min at 20°C. The supernatant fluid was discarded and the pellet re-suspended with 210 µl of PBS (containing $CaCl_2$ and $MgCl_2$). An estimation of the cell concentration was determined using trypan blue solution (0.4%) and a haemocytometer. An appropriate amount of PBS (containing $CaCl_2$ and $MgCl_2$) was used to re-suspend the PMNs at a concentration of $1x10^6$ cell ml^{-1}.

Boyden chamber assay

In vitro cell migration assays were performed using a modified 48-well Boyden Chamber. The lower chamber wells were loaded with 27 µl of chemoattractant fluid such that a slight positive meniscus formed to prevent air bubbles. PVP-free polycarbonate filters with pores 3 µm in diameter were utilised. The upper wells were filled with 45 µl of cell suspension at a concentration of $1x10^6$ cells ml^{-1}in Dulbecco's PBS with $CaCl_2$ and $MgCl_2$. The Boyden chamber was then placed in an incubator with a 5% CO_2 atmosphere at 37°C for 30 min. Following incubation, the filter was removed and placed, with the 'non-migrated cell side' facing downwards, in a Petri dish containing 70% ethanol for 7 min, then in another containing distilled H_2O for 3 min - in order to reduce the degree of adhesion between the non-migrated cells and the filter. Non-migrated cells were then removed by carefully drawing the filter over a wiper blade. The filter was allowed to air dry before fixation and staining with Diff-Quik® stain set. Finally, the filter was mounted onto a microscope slide using xylene and DPX. The migrated cells were counted in ten non-overlapping fields (x40 magnification) with a light microscope and the order in which wells were counted was randomised. The migrated cells were counted by one scorer. The order in which wells were counted was randomized prior to each experiment.

The set-up of the Boyden chamber and the incubation time of the cells varied depending on whether stimulation or inhibition of migration was being investigated according to the following protocols:

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Protocol I. Stimulation of neutrophil migration

Neutrophils, at a concentration of $1x10^6$ cells.ml^{-1}, were loaded into the upper wells of the Boyden chamber, while the lower wells contained test compound. 1 μM fMLP acted as positive control.

Protocol II. Inhibition of neutrophil migration

Neutrophils were pre-incubated with test compound(s) for 30 min at 37ºC in a water bath before loading into the upper wells. The lower wells contained the corresponding concentration of test compound and 1 μM fMLP. This arrangement ensures that the only concentration gradient present is that generated by fMLP as it diffuses through the pores in the filter.

Analysis of data

The mean number of neutrophils migrated in response to 1 μM fMLP in the presence of test compounds was normalised against the mean number of migrated neutrophils elicited by 1 μM fMLP with test compound vehicle (0.01% DMSO), the number of migrated cells in the presence of basal (fMLP vehicle only) was subtracted, as illustrated in equation 1:

$$\left(\frac{\#\text{ of cells migrated}\left[(\text{test compound} + \text{fMLP}) - (\text{basal})\right]}{\#\text{ of cells migrated}\left[(\text{fMLP} + \text{vehicle}) - (\text{basal})\right]} \right) X\ 100 = \text{migration (\% of fMLP-induced migration)}$$

All data are expressed as means ± s.e.mean. IC_{50} and E_{max} values, together with the s.e.mean or 95% confidence intervals were calculated by non-linear regression analysis using the equation for sigmoidal concentration-response curve (GraphPad Prism 4). Statistical analyses were performed with GraphPad Prism 4. A one-sample *t*-test was used to compares the % neutrophil migration in the presence of test compounds with 100%, which is the level of migration in the presence of vehicle alone. Analysis of variance (ANOVA) followed by Dunnett's test or Students' paired *t*-test was used to compares the % migration in the presence of vehicle with the presence of antagonists or enzyme inhibitors. A P value <0.05 was considered to be

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significant. In all cases the *n* number given in the text represents individual measurements from separate Boyden chamber experiments performed using neutrophils derived from blood donated by at least 3 different donors.

Results

Stimulation of migration

The ability of various cannabinoids to induce human neutrophil migration was investigated using protocol *I.* (see Methods). The endogenous cannabinoid anandamide (AEA) (Figure 1A) and 2-arachidonoyl glycerol (2-AG) (Figure 1B), induced a modest stimulation of migration of neutrophils that was not concentration related. Neither the synthetic non-selective CB_1/CB_2 receptor agonist, CP55940 nor the CB_2 receptor selective agonist, JWH-133 produced a significant migration of human neutrophils (data not shown). In Figure 1 the data are expressed as the number of neutrophils from the count of ten non-overlapping fields (x40 magnification) in each well. This highlights the variability inherent in using freshly isolated human neutrophils. Over a large number of experiments (n = 103), the neutrophils in each well over ten non-overlapping fields, was 200 ± 25 and 1983 ± 145 in the presence of vehicle (basal) and fMLP (1μM) respectively ($P<0.001$, Student's paired t-test). Because of the inter-donor variability in migration, in subsequent experiments the number of migrated neutrophils was normalised to an fMLP (1μM) control for each donor and the data is expressed as a % of this donor-specific control.

Anandamide (AEA) inhibits human neutrophil migration

After pre-incubation with AEA (protocol *II*, see Methods), neutrophil migration induced by fMLP (1 μM) was significantly attenuated (Figure 2A). AEA also significantly attenuated human neutrophil migration induced by LTB_4 (100 nM) (one sample *t*-test; n = 9) (Figure 2B). In both cases, significant inhibition was observed at concentrations as low as 0.1nM.

Anandamide (AEA) does not affect cell viability

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Using the trypan blue exclusion method, the estimated percentage viability of neutrophils incubated for 30 mins in vehicle (0.01% DMSO) or AEA (100 nM) was 97.2 ± 0.52% and 97.5 ± 0.35%, respectively; these values were not significantly different from one another (P>0.05, Student's unpaired *t*-test; n = 3).

Certain endogenous lipids, phytocannabinoids and related ligands inhibit human neutrophil migration.

Endocannabinoids and endogenous lipids

Next we investigated the effect of various endocannabinoids and other endogenous lipids that have been reported to interact with either the abnormal-CBD receptor (Begg et al, 2005) or GPR55 (Ryeberg et al, 2007) (see Table 2).

The endocannabinods virodhamine and *N*-arachidonoyl dopamine (NADA) significantly inhibited neutrophil migration induced by fMLP (n = 9, Figure 3A). The IC_{50} and E_{max} values for these compounds are shown Table 1.

2-AG (2-arachidonoyl glycerol) and palmitoylethanolamide (PEA) had no significant effect on the neutrophil migration induced by fMLP (n = 9-12, Figure 3A) using protocol *II* (see methods). At concentrations up to 1 μM, *N*-arachidonoyl-L-serine (ARA-S) did not inhibit human neutrophil migration (Figure 3A). However, this compound behaved as an antagonist (see later).

The putative GPR55 agonist (Oka et al, 2007), lysophosphatidylinositol (LPI) did not inhibit human neutrophil migration (Figure 3C).

Phytocannabinoids and related ligands

We also investigate the effects of a range of compounds that are found in *Cannabis Sativa* and structurally-related ligands that have been reported to interact with either the abnormal-CBD receptor or GPR55.

(-)-CBD did not stimulate neutrophil migration (P>0.05, one sample *t*-test; n = 9); the extent of migration being 0.09 ± 0.26 , -0.02 ± 0.18, -0.03 ± 0.07, -0.02 ± 0.24, -0.20 ± 0.13 and 0.25 ± 0.25% of the fMLP control (1 μM) stimulated by 0.01 nM, 0.1 nM,

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1 nM, 10 nM, 100 nM and 1 μM (-)-CBD, respectively (Protocol *I* see methods). In contrast, (-)-CBD significantly inhibited neutrophil migration induced by fMLP (1 μM) (one sample *t*-test; n = 9-15, Figure 3B). Whilst (-)-CBD was highly potent (IC_{50} of 0.45 nM), it had a significantly lower efficacy (E_{max}) than virodhamine (see Table 1). Using the trypan blue exclusion method, the estimated percentage viability of neutrophils incubated for 30 mins in vehicle (vh) (0.01% DMSO) or (-)-CBD (1 μM) was 96.6 ± 0.5% and 96.8 ± 0.3%, respectively, which were not significantly different from one another (P>0.05, Student's unpaired *t*-test; n = 3).

(+)-CBD also significantly inhibited neutrophil migration induced by fMLP (1 μM) (one sample *t*-test; n = 9, Figure 3B), but was ~ 40 fold less potent than it's stereoisomer, (-)-CBD (see Table 1). The close structural analogue of CBD, abnormal-cannabidiol (abn-CBD) significantly inhibited neutrophil migration induced by fMLP (1 μM) (one sample *t*-test; n = 9, Figure 3B). Abn-CBD was ~70 fold less potent than (-)-CBD, but appears to have a higher efficacy (E_{max}) (see Table 1).

The synthetic abn-CBD analogue, O-1602, which is an agonist of the abn-CBD receptor (Járai et al, 1999) and GPR55 (Johns at el, 2007; Ryeberg et al, 2007), inhibited human neutrophil migration with a similar potency to abnormal-CBD (see Table 1, 2 and Figure 3B).

The major psychoactive constituent of cannabis, Δ^9-THC had no significant effect on the neutrophil migration induced by fMLP (1 μM) (one sample *t*-test; n = 9, Figure 3B).

The effect of receptor antagonists of cannabinoid-mediated inhibition of human neutrophil migration.

In the next series of experiments we investigated the receptors underlying the inhibition of human neutrophil migration by cannabinoids and related ligands. The effect of the CB_1 receptor antagonist, SR141, the CB_2 receptor antagonists SR144 and AM630 and the TRPV1 receptor antagonist, capsazepine (CPZ) on the ability of AEA to inhibit fMLP-induced neutrophil migration was tested using protocol *II* (see Methods).

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The inhibition of fMLP-induced neutrophil migration by AEA (100 nM) was significantly attenuated by SR141 (1 μM) ($P<0.001$, one-way ANOVA; n = 9) (Figure 4A). AEA-mediated inhibition of fMLP-induced migration was significantly enhanced in the presence of the CB_2 antagonist, SR144 (100 nM) ($P<0.001$, one-way ANOVA; n = 9). Capsazepine (CPZ) had no significant effect on the ability of AEA (100 nM) to inhibit fMLP-induced neutrophil migration ($P>0.05$, one-way ANOVA; n = 9) (Figure 4A). Because SR144 (100 nM) enhanced the inhibition of fMLP-induced neutrophil migration by AEA (100 nM), the effect of a structurally distinct CB_2 receptor antagonist, AM630 was also investigated. AM630 also significantly enhanced the ability of AEA (100 nM) to inhibit fMLP-induced neutrophil migration ($P<0.001$, Student's unpaired *t*-test; n = 9) (Figure 4B). Neither SR141 (1 μM), SR144 (100 nM), CPZ (1 μM) nor AM630 (100 nM) alone had any significant effect on fMLP-induced migration (one sample *t*-test; n = 9) (Figure 4D).

The ability of AEA to inhibit fMLP-induced neutrophil migration in the presence of the CB_1 receptor antagonist, SR141716A (SR141) (1 μM) was further investigated. Again, SR141 (1 μM) alone had no significant effect on fMLP-induced migration (one sample *t*-test; n = 3), the migration being 100.6 ± 2.03% ($P>0.05$) of fMLP with vehicle. The log concentration-response curve for AEA appears to be bi-phasic (Figure 4C); in the presence of 1 μM SR141 there was a rightward shift in the first phase of the log concentration-response curve for AEA (Figure 4C).

Inhibition of fMLP-induced migration virodhamine (10 nM) was significantly attenuated in the presence of the CB_1 antagonist, SR141 (1 μM) ($P<0.05$, one-way ANOVA; n = 12, Figure 5A). In contrast, virodhamine (10 nM) mediated inhibition of fMLP-induced migration was enhanced in the presence of the CB_2 antagonists, SR144 (100 nM) and AM630 (1 μM) ($P<0.01$, one-way ANOVA; n = 12, Figure 5A). The TRPV1 receptor antagonist, capsazepine (CPZ) had no effect on virodhamine-mediated inhibition of migration ($P>0.05$, one-way ANOVA; n = 12, Figure 5A).

In the presence of the CB_2 receptor antagonist SR144, the synthetic CB_1/CB_2 receptor antagonist, CP55940 significantly inhibited fMLP-induced inhibition of human neutrophil migration, an effect that was not seen in the absence of the antagonist (Figure 5B).

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_N_-arachidonoyl L-serine (ARA-S) antagonises the inhibition of human neutrophil migration by virodhamine and abnormal-CBD.

As shown in figure 3A, at concentrations up to 1 μM, *N*-arachidonoyl-L-serine did not inhibit human neutrophil migration. However, at 1 μM this compound significantly attenuated the inhibition of fMLP stimulated human neutrophil migration induced by anandamide ($P<0.001$, Students unpaired test; Figure 6A). Furthermore, this compound abolished the inhibition of human neutrophil migration induced by abnormal-CBD (Figure 6B) and significantly reduced the E_{max} for inhibition of migration by virodhamine to 45% (95% confidence limits, 42 – 47; Figure 6C).

Discussion

Our investigation revealed an intriguing pharmacology underlying the inhibition of human neutrophil chemotaxis indicating that certain endocannabinoids and phytocannabinoids are potent inhibitors of human neutrophil migration.

CB_1 receptor expression in human neutrophils is low (Galiégue et al, 1995) and the weight of evidence indicates that cannabinoid CB_1 receptors do not play a role in the cannabinoid-mediated inhibition of induced human neutrophil migration observed in this study. Firstly, a number of compounds display potent inhibitory behaviour that is inconsistent with their pharmacology at CB_1 receptors; virodhamine is a CB_1 receptor antagonist (Porter et al, 2002) and (-)-CBD has low affinity for the CB_1 receptor (Thomas et al, 1998; Bisogno et al, 2001); 2-AG and Δ^9-THC, which are both agonists of the CB_1 receptor (Pertwee and Ross, 2002), do not inhibit neutrophil migration. It has been demonstrated that the CB_1 receptor antagonist, SR141 can antagonise non-CB_1 receptors at concentrations in the micromolar range (Begg et al, 2005; Drmota et al, 2004), therefore we made a conscious decision to employ a concentration of 1 μM. The inhibition of neutrophil migration by virodhamine was attenuated by 1 μM SR141. The inhibition of neutrophil migration by anandamide appears to be bi-phasic in nature and the first phase of this inhibition by the CB_1 receptor antagonist 1 μM SR141. The nature of the log-concentration response curve

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for AEA suggests that more than one target may be involved in the inhibition of human neutrophil migration by this endocannabinoid. Taken together, these results are consistent with a role for a non CB_1-receptor in mediating the effect of these endocannabinoids (MacLennan et al, 1998; Pertwee, 1999; Begg et al, 2005).

Kurihara *at al* (2006) recently found surface expression of CB_2 receptors on neutrophil-like HL60 cells and human neutrophils. In our investigation, a role for CB_2 receptors in the modulation of neutrophil migration was implied by the fact that two, structurally distinct, CB_2-selective antagonists significantly enhanced the cannabinoid-mediated inhibition. There is considerable evidence that cannabinoid CB_1 and CB_2 receptors exist in a conformation that is pre-coupled to the G-protein Pertwee, 2005). Both SR144 and AM630 are CB_2 receptor inverse agonists (Bouaboula et al, 1999; Ross et al, 1999) and, as such, they bind with a high affinity to the pre-coupled CB_2 receptors. CB_2 receptors on human neutrophils may be auto-activated, exerting high basal levels of CB_2 receptor-mediated signalling; thereby precluding inhibition mediated by certain other receptors. Furthermore, auto-activation may underlie the lack of significant stimulation of neutrophil migration by the CB_2 receptor agonists 2-AG, CP55940, JWH-133 and JWH015 (this study and Kurihara et al 2006). In line with our findings, Lunn et al (2006) have demonstrated that CB_2 receptor inverse agonists inhibit leukocyte migration both *in vivo* and *in vitro* and the level of effect is proportional to the degree of inverse efficacy.

Particularly pertinent to our neutrophil study is the non-CB_1, non-CB_2 pharmacological target named the 'abnormal-cannabidiol' (abn-CBD) receptor, which is antagonised by SR141, but only at concentrations considerably higher than those predicted from its CB_1 receptor affinity (Begg et al, 2005). Evidence for the existence of abn-CBD receptors initially emerged from studies in certain blood vessels, which relax in response to AEA and abn-CBD; an effect that is maintained in vessels from $CB_1^{-/-}$ mice (Jarái et al, 1999). Notably, neither 2-AG not Δ^9-THC relax these vessels (Ho and Hiley, 2003). Whilst cannabidiol (CBD) acts as an antagonist of the abn-CBD receptor in certain vessels, in others CBD appears to act as an agonist, sharing the ability of abn-CBD to relax vessels. In addition, virodhamine and *N*-arachidonoyl dopamine (NADA) induce a relaxation of mesenteric arteries, being more potent than either AEA or abn CBD (Ho and Hiley, 2004; O'Sullivan et al, 2004; Begg et al,

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2005). Probably of the greatest relevance to our data are studies in microglial cells which provide robust evidence for a role of Abn-CBD receptors in cell migration (Walter et al, 2003). Thus, 2-AG triggers microglial cell migration by acting through CB_2 and abn-CBD receptors. CBD acts as a partial agonist thereby inducing migration alone but also attenuating migration induced by a full agonist. Stimulation of microglial cell migration by cannabinoids is antagonised by high but not low concentrations of SR141 (Franklin and Stella, 2003). It is apparent that there are certain parallels between the pharmacology that we observed in human neutrophils and pharmacology of the abn-CBD receptor, which is summarised in Table 2. Firstly, the effects of both AEA and virodhamine are sensitive to antagonism by 1 μM SR141 which is line with the proposed affinity of the CB_1 receptor antagonist for the abn-CBD receptor (Begg et al, 2005). Secondly, agonist efficacy and potency closely parallel that previously obtained for the abn-CBD receptor in blood vessels and microglial cells (see Table 2); of the phytocannabinoids and structurally-related ligands, (-)-CBD has low efficacy, but is more potent than both (+)-CBD and abn-CBD; O-1602, an analogue of abn-CBD in which the pentyl side chain was shortened to a methyl group, is an agonist and Δ^9THC is inactive; of the endocannabinoids and related lipids, anandamide, *N*-arachidonoyl dopamine (NADA) and virodhamine are active, the later having the highest potency; palmitoylethanolamide is inactive. Perhaps the more intriguing finding in the current study is the antagonism of various cannabinoids by *N*-arachidonoyl L-serine (ARA-S), a brain constituent previously shown to be an agonist at the abn-CBD receptor (Millman et al, 2005). Here we find that this endogenous compound attenuates the inhibition of human neutrophil migration by AEA, virodhamine and abn-CBD. It is also notable that, whilst 1μM *N*-arachidonoyl L-serine (ARA-S) abolishes the effect of abn-CBD, it reduces the E_{max} value of virodhamine. Thus, the effect of virodhamine may involve more than one receptor, only one of which is blocked by ARA-S. Alternatively, ARA-S may be acting as an allosteric inhibitor of the target receptor; a reduction in E_{max} is characteristic of an allosteric inhibitor. In line with our findings, in a recent abstract Zhang et al (2006) reports that abn-CBD inhibits angiogenesis, an effect that is antagonised by ARA-S. This raises two possibilities: either this endogenous lipid mediator is a partial agonist at the abn-CBD receptor and thereby also acts as

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antagonist in certain conditions; or the effects observed in this study are due, at least in part, to activation of another, perhaps related target.

Recent publications (Ryberg et al, 2007; Johns et al, 2007) have emerged suggesting certain cannabinoid ligands interact with an orphan receptor GPR55 and that this, and possibly other orphan receptors, may account for the pharmacological and functional evidence for some novel CB receptors (see Baker at al, 2006). Whilst it has been suggested that the abn-CBD receptor is, in fact GPR55, there are notable differences in the reported pharmacology of these two targets as summarised in Table 2 (Mackie and Stella, 2005; Baker et al, 2006). It is particularly notable that whilst abn-CBD has high affinity for GPR55, it retains the vasodilator effects in mice lacking GPR55, the implication being that this atypical cannabinoid activates more additional novel receptors (Johns et al, 2007; see Hiley & Kaup, 2007). With regard to human neutrophils, it is noteworthy that GPR55 is expressed in spleenic tissue and that virodamine, which was the highest efficacy and potency compound in our study, is also reported to be a high efficacy agonist of GPR55 (Ryberg et al, 2007). Reports suggest that GPR55 and related orphan receptors are G_{13}-coupled and can activate the Rho pathway (Ryberg et al, 2007), which plays an important role in the regulation of myosin light chain phosphorylation and subsequent cytoskeletal-dependent locomotion (Buhl et al, 1995; Kozasa et al 1998). Therefore, as a consequence of its G_{13}-coupling it appears that inverse agonism of GPR55 would be necessary in order to deliver an anti-migratory signal to the cellular locomotory machinery. However, there is considerable evidence for cross-desensitation of GPCRs leading to inhibition of fMLP-induced migration (Ali et al, 1999), thereby affording speculation that agonists of this receptor may be inhibitory. Kurihara *et al* (2006) have recently demonstrated that the CB_2 receptor plays a role in human neutrophil migration by modulating RhoA activation; one might speculate that cross-talk between the CB_2 receptor and the novel target located in human neutrophils occurs at the level of Rho signalling. Some controversy surrounds the pharmacology of GPR55; Oka et al, (2007) report that lysophosphatidylinositol (LPI), but not cannabinoid ligands, induce ERK phosphorylation in GPR55-expressing cells. Here we report that LPI does not inhibit human neutrophil migration.

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Our data suggest that in human neutrophils, there is cross-talk/negative co-operativity between the cannabinoid CB_2 receptor and a novel receptor such that inhibition of pre-coupled CB_2 receptors enhances the inhibition observed in response to activators of this receptor. This is in line with the positive co-operatively observed between the CB_2 and abn-CBD receptor in microglial cells (Walter et al, 2003). A realistic explanation for the apparent bidirectional co-operativity exhibited between these two receptors is a difference in underlying signal transduction depending on cell type. Taken together, our data provide the first evidence that human neutrophil migration can be modulated by a novel receptor and that, in these cells, the CB_2 receptor exerts negative co-operativity upon this receptor.

This study reveals that certain endogenous lipids, phytocannabinoids and related ligands are potent inhibitors of human neutrophil migration and implicates a novel pharmacological target distinct from cannabinoid CB_1 and CB_2 receptors; this target is antagonised by the endogenous compound, *N*-arachidoloyl L-serine. These findings corroborate the emerging clinical and animal model data demonstrating that the non-psychoactive phytocannabinoid, CBD and its structural analogues are effective in alleviating arthritis (Blake et al, 2006; Malfait et al 2000). Furthermore, our findings have implications for the potential pharmacological manipulation of elements of the endocannabinoid system for the treatment of various inflammatory conditions.

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Footnotes

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Figure Legends

Figure 1: Histograms showing the number of neutrophils induced to migrate by (A) anandamide (AEA) and (B) 2-arachidonoyl glycerol (2-AG). The data represent the mean number of neutrophils migrated ± s.e.mean (n = 3 - 9). * P<0.05, **P<0.01, one-way ANOVA.

Figure 2: Histograms showing neutrophil migration (A) induced by fMLP (1 μM) following pre-incubation with AEA (B) induced by LTB_4 (100 nM) following pre-incubation with increasing concentrations of AEA. The data represent the mean number of neutrophils migrated ± s.e.mean (n = 9) as a percentage of the migration induced by fMLP or LTB_4 in the presence of vehicle (0.01% DMSO). ***P<0.001, **P<0.01, *P<0.05, one-sample t-test.

Figure 3: Log concentration-response curves for neutrophil migration induced by 1μM fMLP in the presence of various ligands (response calculated using equation 1, see methods) (A) endocannabinoids and related endogenous lipids; AEA (anandamide), 2-AG (2-arachidonoyl glycerol), PEA (palmitoylethanolamide), NADA (*N*-arachidonoyl dopamine), ARA-S (*N*-arachidonoyl-L-serine) (B) phytocannabinoids and their analogues; CBD (cannabidiol), Abn-CBD (Abnormal cannabidiol), O-1602, Δ^9THC (Δ^9 tetrahydrocannabinol). (C) Histogram showing the effect of lysophosphatidylinositol (LPI). Data is the mean ± s.e.mean (n = 3 - 12).

Figure 4: Histogram showing neutrophil migration induced by fMLP (1 μM) following pre-incubation with: (A) AEA (100 nM) + vehicle, AEA (100 nM) + SR141 (1 μM), AEA (100 nM) + SR144 (100 nM), and AEA (100 nM) + CPZ (1 μM). AEA (100 nM) significantly inhibits († P<0.05, one sample *t*-test) migration induced by fMLP (1 μM). In the presence of SR141 (1 μM), the inhibition is significantly attenuated (***P<0.001, one-way ANOVA). In the presence of SR144 (100 nM), the inhibition is significantly enhanced (***P<0.001, one way ANOVA). (B) AEA (100 nM) + vehicle or AEA (100 nM) + AM630 (100 nM). AEA (100 nM) significantly inhibits ††† P<0.001, one sample *t*-test) neutrophil migration induced by fMLP (1 μM). In the presence of AM630 (100 nM), the inhibition is significantly

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enhanced (*** P<0.001, Student's unpaired *t*-test). (C) Log concentration-response-curves showing the percentage of fMLP (1 μM)-induced migration in the presence of increasing concentrations of AEA with either vehicle or 1 μM SR141 (D) pre-incubation with SR141 (1 μM), SR144 (100 nM), CPZ (1 μM) or AM630 (100 nM). None of these antagonists have any significant (P>0.05, one sample *t*-test) effect on neutrophil migration induced by 1 μM fMLP. The data represent the mean number of neutrophils migrated ± s.e.mean (n = 9) as a percentage of the migration induced by 1 μM fMLP in the presence of vehicle (0.01% DMSO).

Figure 5: (A) Histogram showing neutrophil migration induced by fMLP (1 μM) following pre-incubation with: virodhamine (10 nM), virodhamine + SR141 (1 μM), virodhamine (10 nM) + SR144 (100 nM), virodhamine (10 nM) + CPZ (1 μM), and virodhamine (10 nM) + AM630 (100 nM). Virodhamine (10 nM) significantly inhibits († P<0.05, one sample *t*-test) migration induced by fMLP (1 μM). Asterisks represent P values calculated from one-way ANOVA compared to virodhamine alone. The data represent the mean number of neutrophils migrated ± s.e.mean (n = 9) as a percentage of the migration induced by 1 μM fMLP in the presence of vehicle (see equation 1 in methods). (B) Histogram showing neutrophil migration induced by fMLP (1 μM) followed by pre-incubation with CP55950 at 100 nM and 1 μM, in the absence and presence of SR144 (100nM). CP55940 (1 μM) significantly inhibits (†† P<0.01, one sample *t*-test) neutrophil migration induced by fMLP (1 μM). In the presence of SR144 (100 nM), both 100nM and 1 μM inhibit fMLP induced migration (††† P<0.001, one sample *t*-test). The inhibitory effect of both concentrations of CP55940 is significantly different in the presence of the antagonist (** P<0.01,*** P<0.001, Student's unpaired *t*-test).

Figure 6: Histogram showing neutrophil migration induced by fMLP (1 μM) following pre-incubation (A) with either vh (0.01% DMSO) or *N*-arachidonoyl L-serine (ARA-S) (1 μM) prior to subsequent pre-incubation with AEA. AEA significantly inhibits († P<0.05, one sample *t*-test) migration induced by fMLP (1 μM). The migration induced by 1 μM fMLP in the presence of AEA + Vh (0.01% DMSO) is significantly lower than compared with AEA + ARA-S (** P<0.001, Student's unpaired *t*-test) (B) with either vh or ARA-S (1 μM) prior to subsequent

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pre-incubation with abnormal-CBD (Abn-CBD) (C) with either vh or ARA-S (1 μM) prior to subsequent pre-incubation with virodhamine. The data represent the mean number of neutrophils migrated ± s.e.mean (n = 9 - 12) calculated using equation 1, see methods.

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Table 1: Inhibition of fMLP-induced migration of human neutrophils by cannabinoids. IC_{50} and E_{max} values (% inhibition of fMLP induced migration) were calculated from sigmoidal concentration-response curves constructed in GraphPad Prism 4. The data represent the mean with 95% confidence limits, n = 3 – 9.

Compound	***IC_{50} (nM) (95% confidence limits)***	***Emax (%) (95% confidence limits)***
Endogenous Lipid Mediators		
Virodhamine	0.18 (0.17 – 0.19)	86.3 (85.7 – 86.8)
N-Arachidonoyl Dopamine (NADA)	8.80 (4.7 – 16.2)	64.0 (56.6 – 71.5)
Anandamide (AEA)	0.14 (0.12 – 0.17)*	54.0 (53.0 – 55.2)*
2-Arachidonoyl Glycerol (2-AG)	>1000	-
N-Arachidonoyl Serine (ARA-S)	>1000	-
Palmitoylethanolamide (PEA)	>1000	-
Lysophosphatidylinositol (LPI)	>1000	-
Phytocannabinoids and analogues		
(-)-Cannabidiol (CBD)	0.45 (0.39 – 0.53)	59.5 (58.0 – 61.0)
(+)-Cannabidiol (CBD)	18.8 (15.4 – 23.0)	70.0 (67.1 – 72.8)
Abnormal-CBD	33.1 (30.0 – 36.4)	69.9 (68.6 – 71.3)
O-1602	32.6 (24.1 – 44.2)	66.6 (64.1 – 69.1)
Δ^9THC	>1000	-

* The concentration-response curve to anandamide is bi-phasic, these values represent the first phase of inhibition.

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Table 2: A comparison of the profile of various compounds as inhibitors of human neutrophil migration (this study) with the reported pharmacology of these ligands at abnormal-CBD receptors and the orphan receptor GPR55.

Compound	***Inhibition of Neutrophil Migration***	***Abnormal-CBD Receptor***	***GPR55***
Endogenous Lipid Mediators			
Virodhamine	+++	++[a]	++ [f]
N-Arachidonoyl Dopamine (NADA)	++	++ [a]	n.d
Anandamide (AEA)	+++	+ [a]	++ [f]
2-Arachidonoyl Glycerol (2-AG)	0	0 [a]	++ [f]
N-Arachidonoyl Serine (ARA-S)	Antagonist	++[d] Agonist [b] Antagonist [c]	n.d
Palmitoylethanolamide (PEA)	0	0[d]	++ [f]
Lysophosphatidylinsoitol (LPI)	0	n.d	++ [h*]
Phytocannabinoids and Related Ligands			
(-)-Cannabidiol (CBD)	+++ Partial agonist	+ [a] /++[d] Partial agonist	++ [f] Antagonist
(+)-Cannabidiol (CBD)	++	n.d	n.d
Abnormal-CBD	++	++[d] + [a]	++ [g] + [f]
O-1602	++	+ [e]	++ [g] ++ [f]
Δ^9THC	0	0 [a,d]	++ [f]
Others			
CP55940	++	0 [a]	++[f]
SR141716A	Antagonist	Antagonist [a,d]	Partial agonist [i]

The symbols represent the potency or affinity of compounds being in the pM range (+++), nM range (++), µM range (+) or not determined (n.d.). References: [a] Begg et al (2005) ; [b] Millman et al, (2005) ; [c] Zhang et al, (2006) ; [d] Walter et al, (2003) ; [e] Járai et al, (1999); [f] Ryeberg et al (2007) ; [g] Johns et al (2007); [h] Oka et al (2007) ; [i] Drmota et al, (2004).* Note that Oka et al (2007) report that cannabinoid ligands do not activate GPR55.

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Figure 1

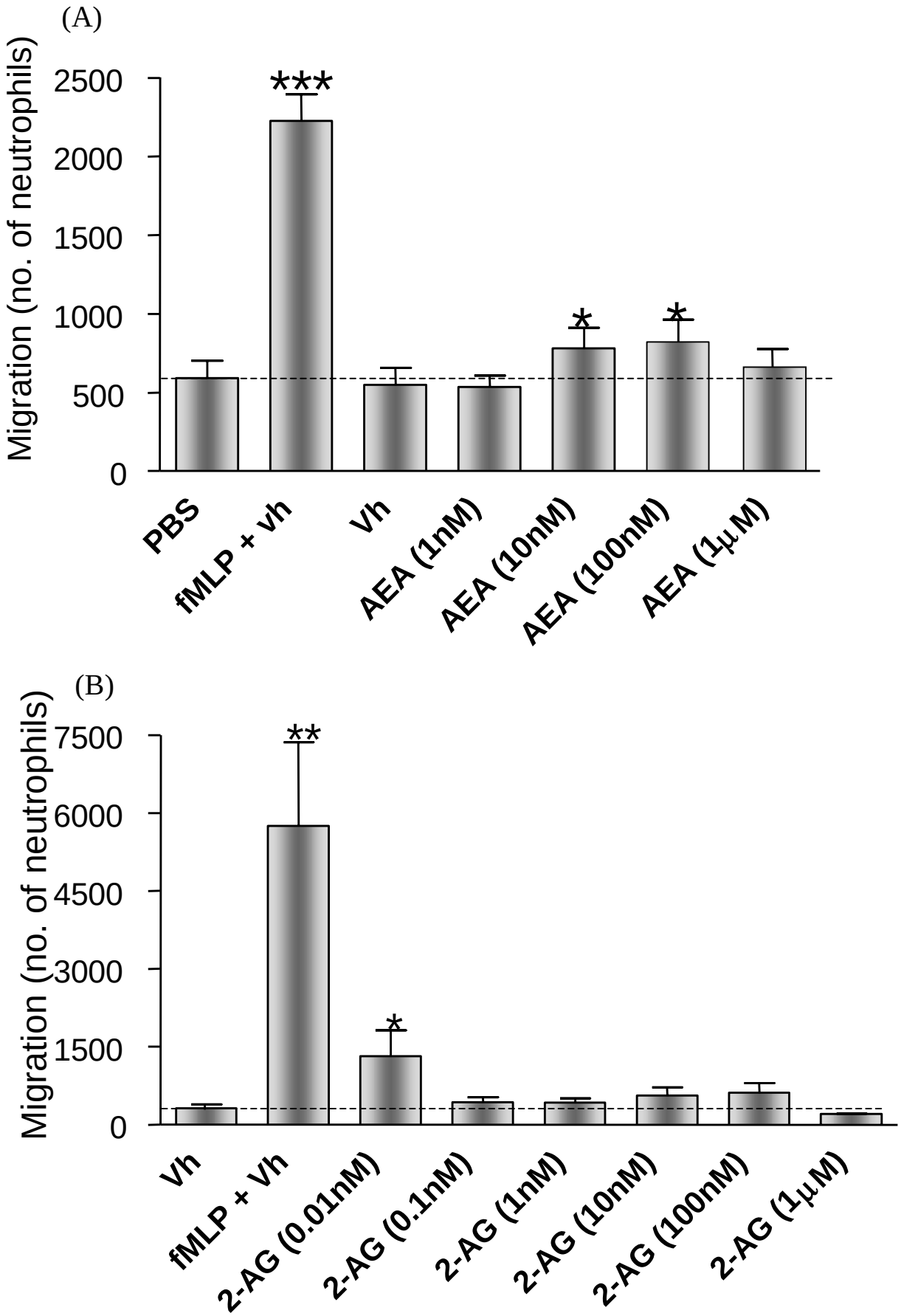

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Figure 2

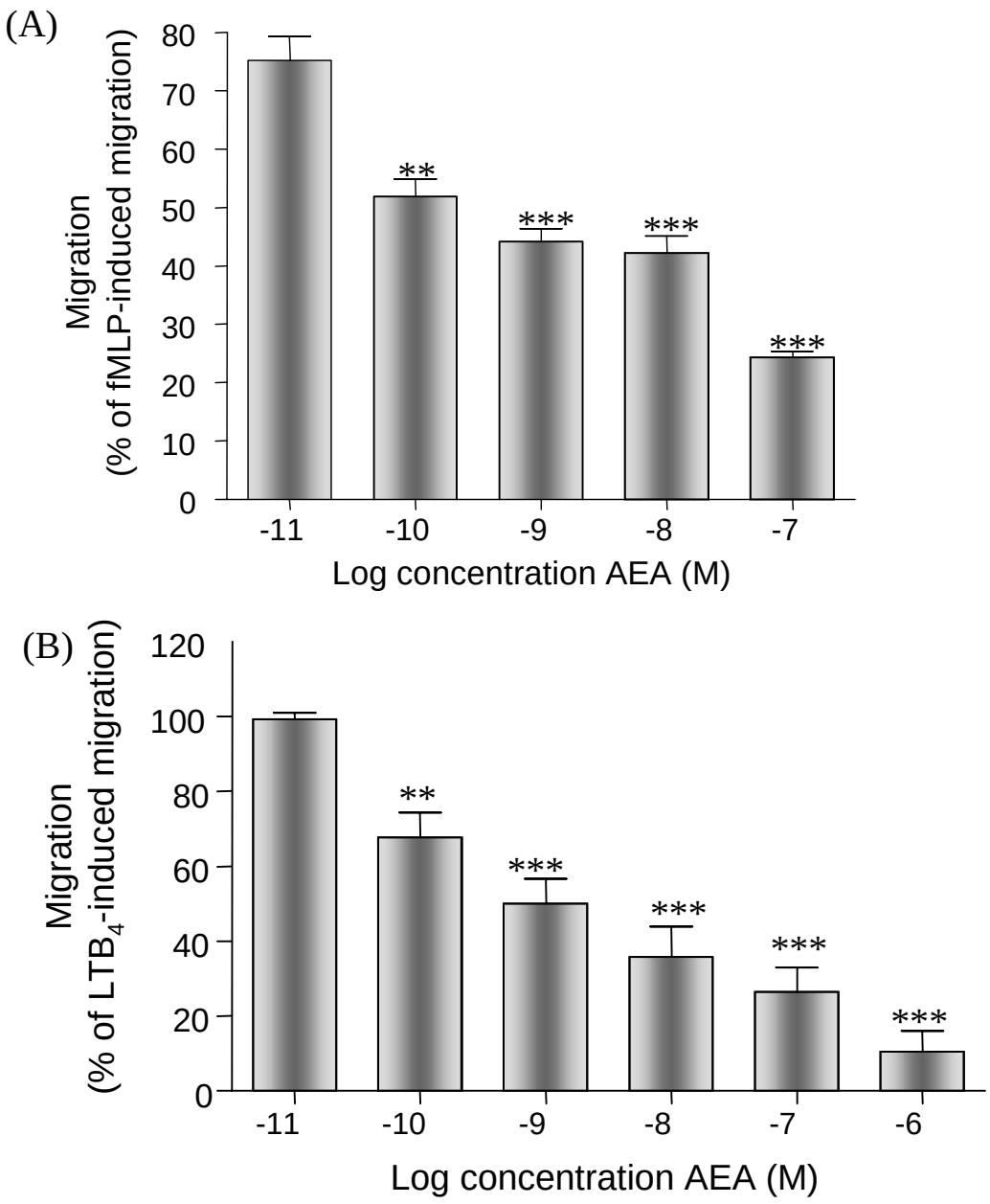

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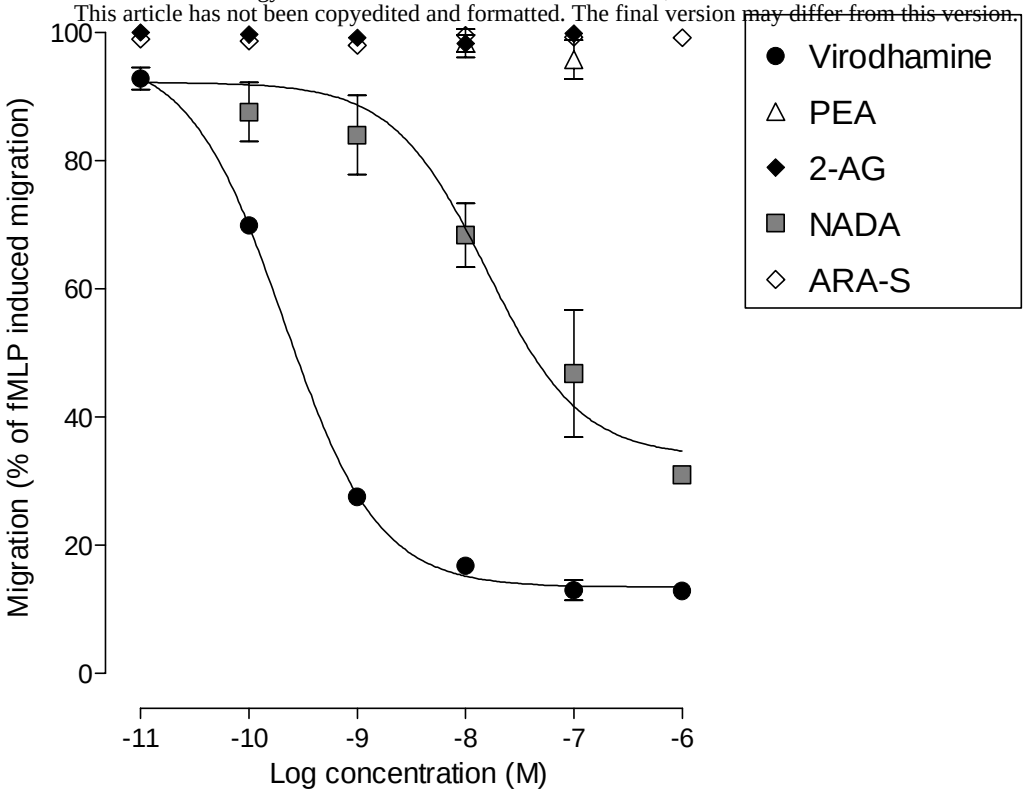
Figure 3

(A)



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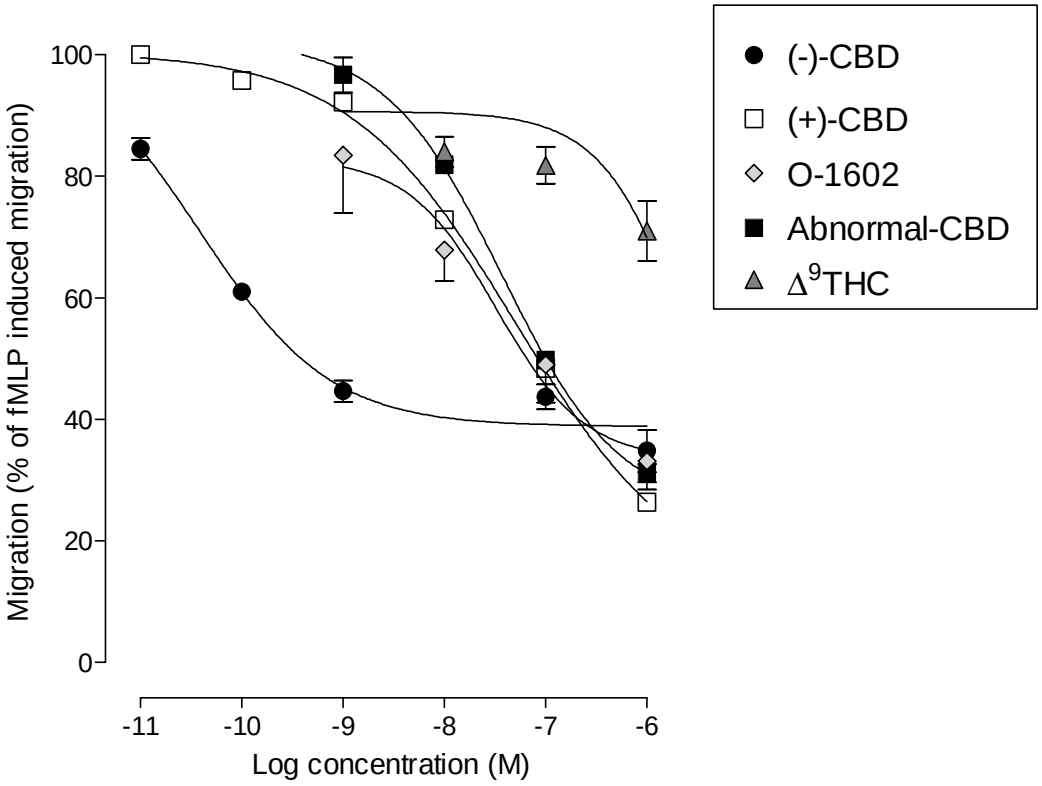

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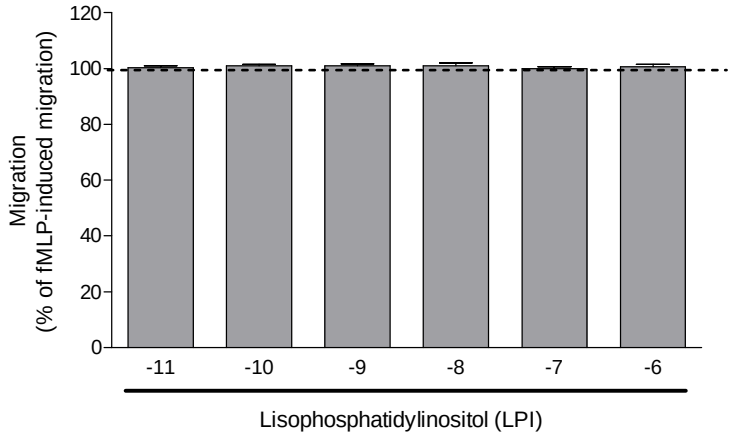

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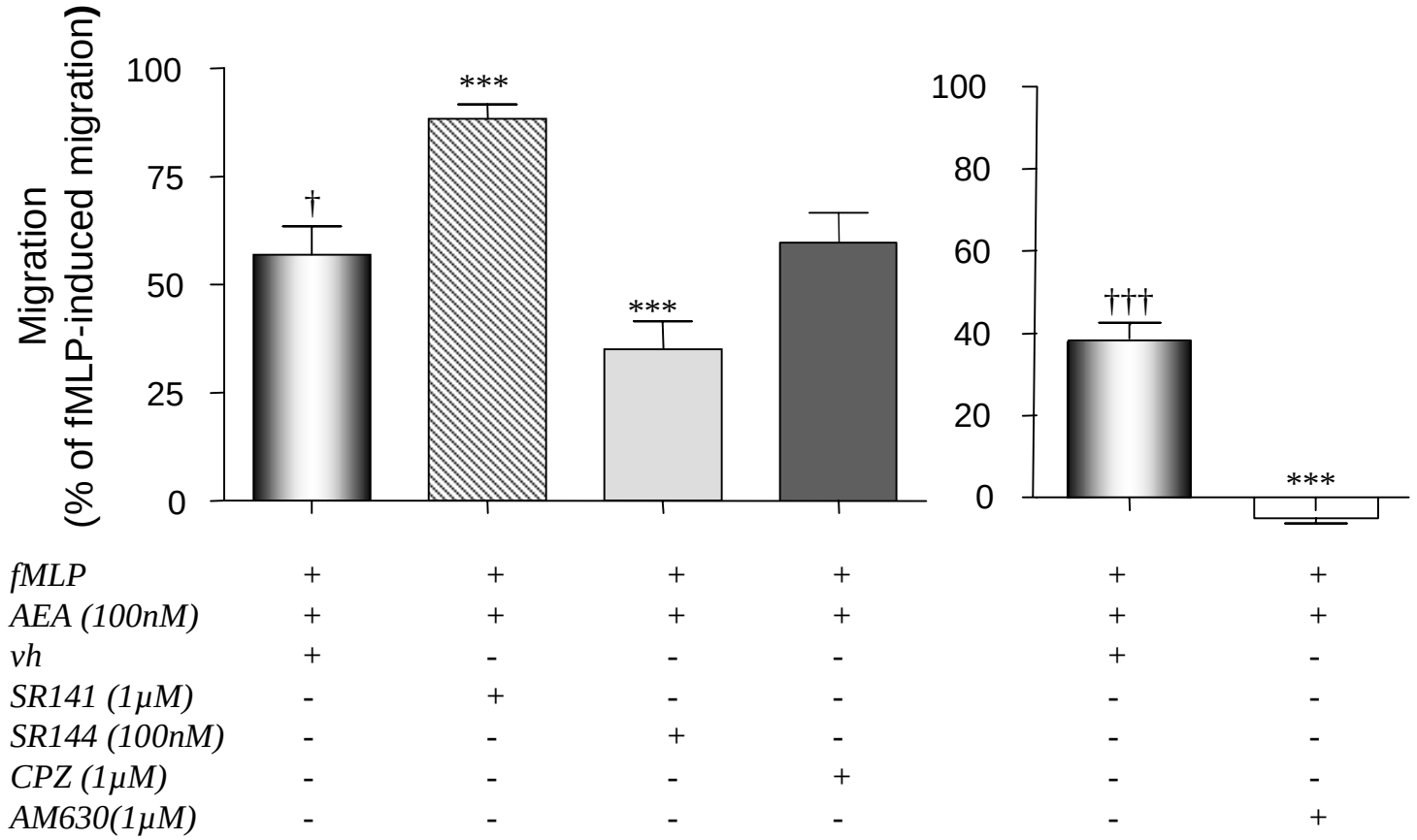
(A)

(B)



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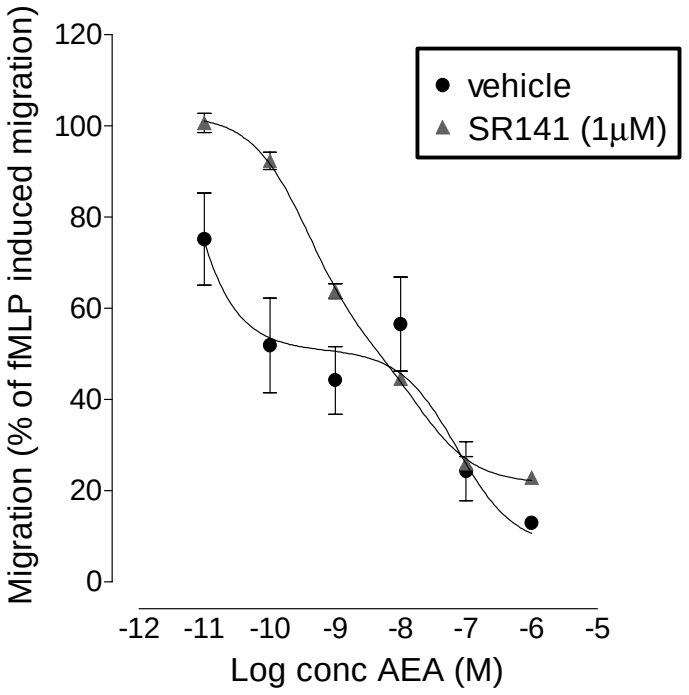

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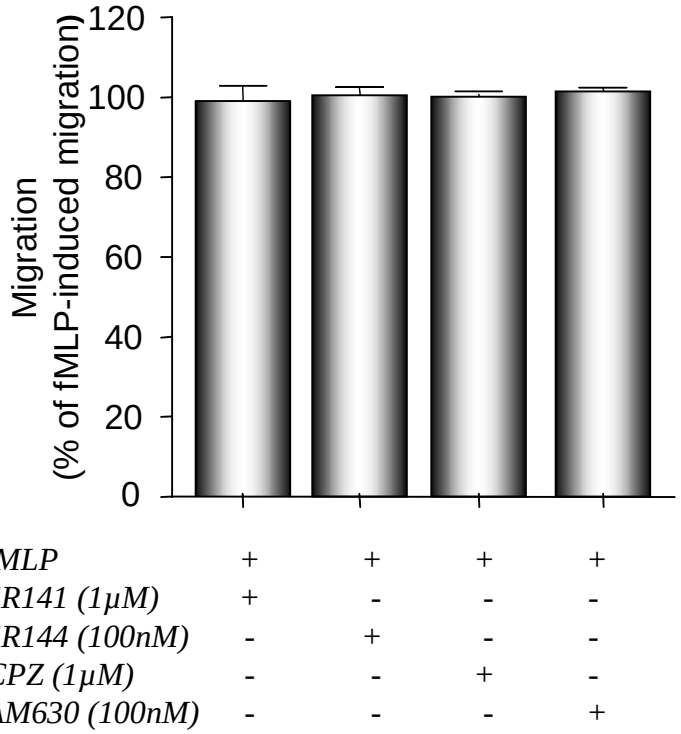

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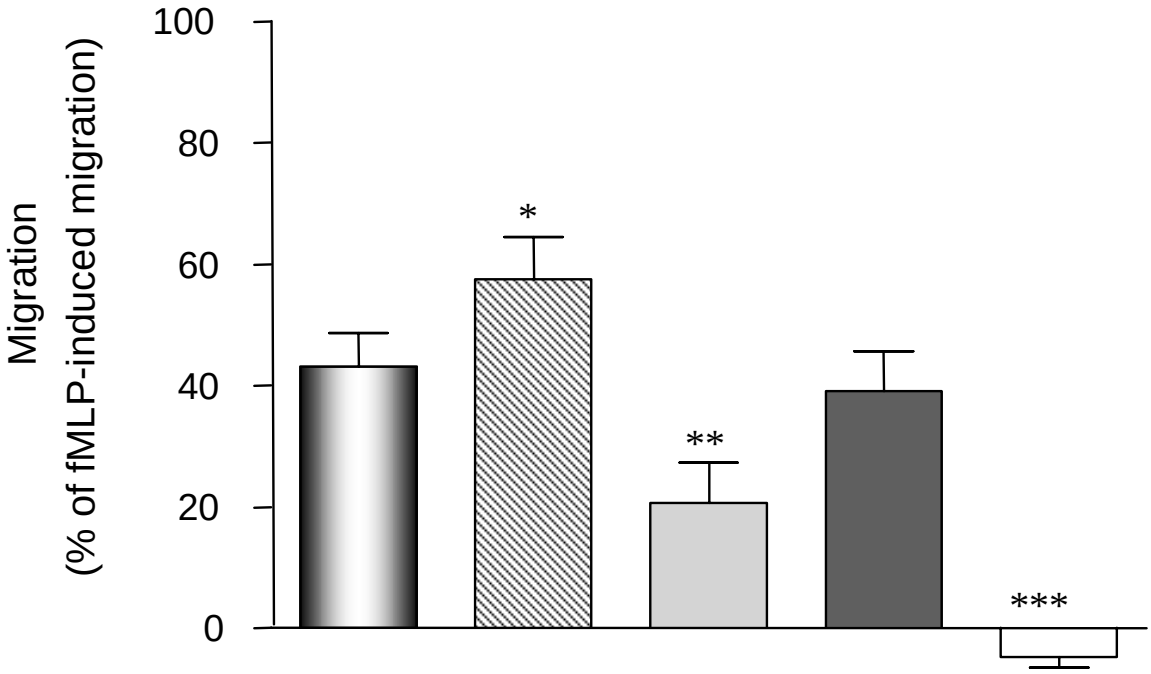
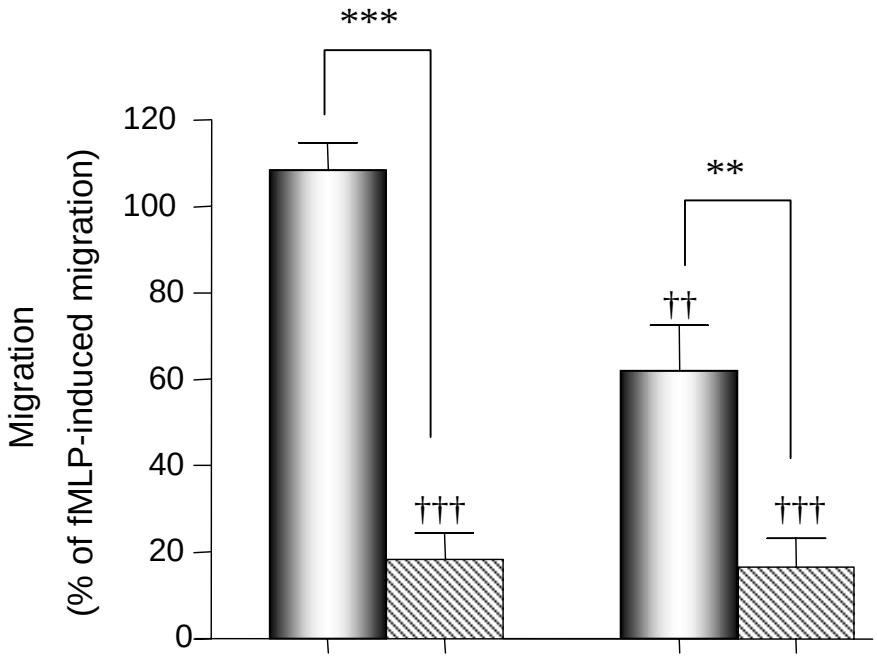


fMLP	+	+	+	+	+
virodhamine (100nM)	+	+	+	+	+
vh	+	-	-	-	-
SR141 (1µM)	-	+	-	-	-
SR144 (100nM)	-	-	+	-	-
CPZ (1µM)	-	-	-	+	-
AM630(1µM)	-	-	-	-	+

(B)



fMLP	+	+	+	+
CP55 (100nM)	+	+	-	-
CP55 (1µM)	-	-	+	+
SR144 (100nM)	-	+	-	+

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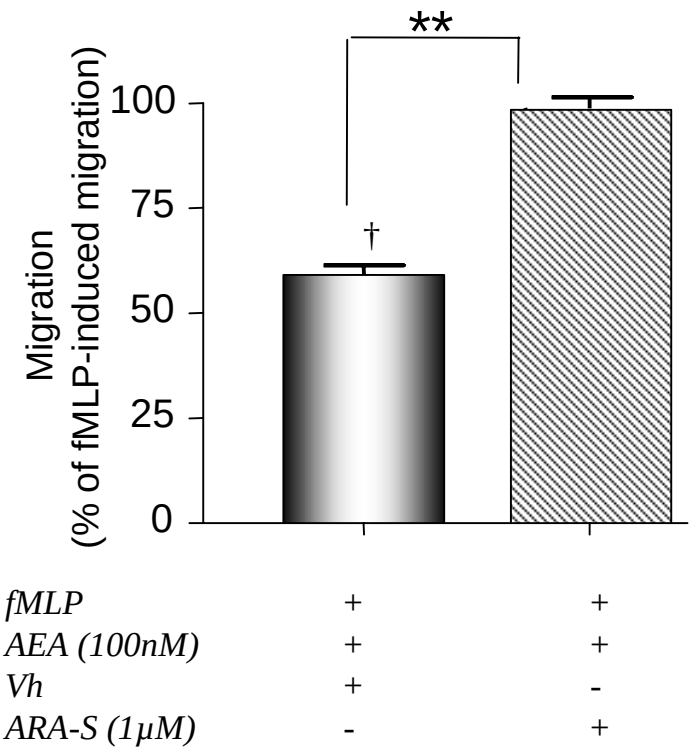
Figure 6

(A)



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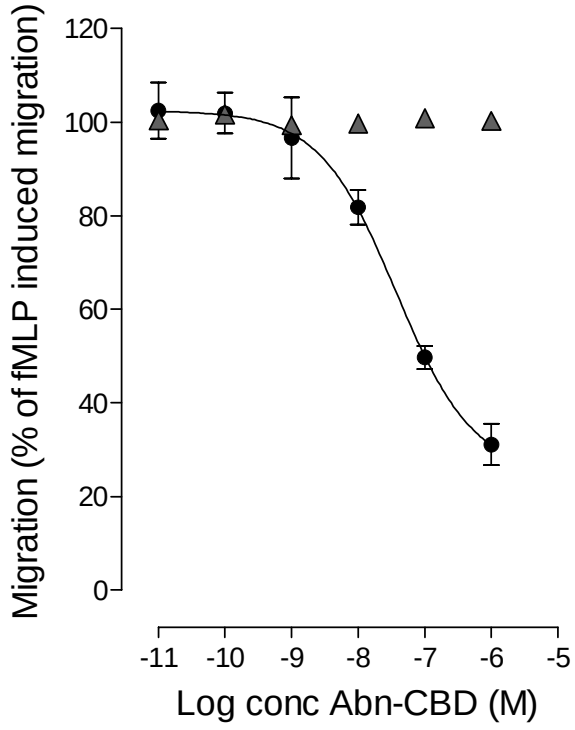

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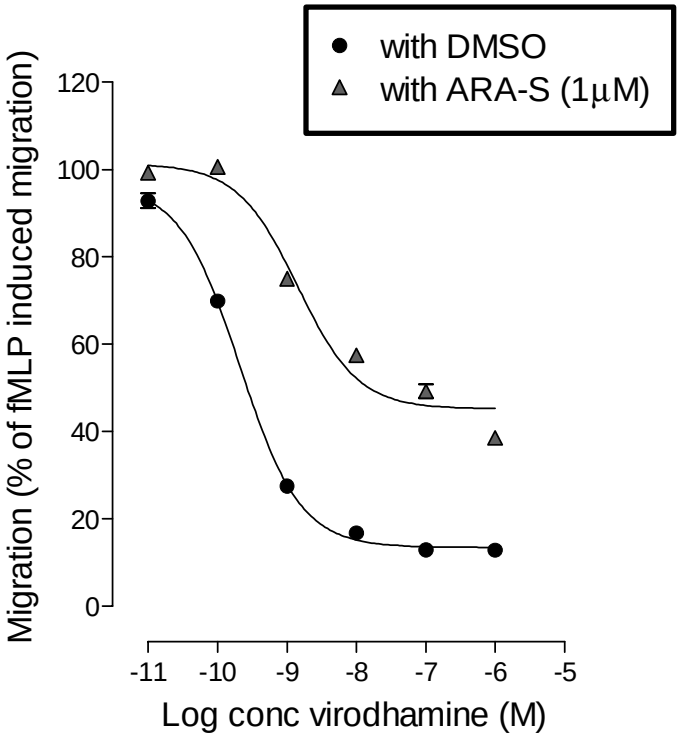

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