



Monoacylglycerol lipase regulates 2-arachidonoylglycerol action and arachidonic acid levels

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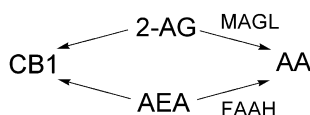
ABSTRACT

The structure–activity relationships of organophosphorus (OP) and organosulfur compounds were examined in vitro and in vivo as inhibitors of mouse brain monoacylglycerol lipase (MAGL) hydrolysis of 2-arachidonoylglycerol (2-AG) and agonist binding at the CB1 receptor. Several compounds showed exceptional potency toward MAGL activity with IC₅₀ values of 0.1–10 nM in vitro and high inhibition at 10 mg/kg intraperitoneally in mice. We find for the first time that MAGL activity is a major in vivo determinant of 2-AG and arachidonic acid levels not only in brain but also in spleen, lung, and liver. Apparent direct OP inhibition of CB1 agonist binding may be due instead to metabolic stabilization of 2-AG in brain membranes as the actual inhibitor.

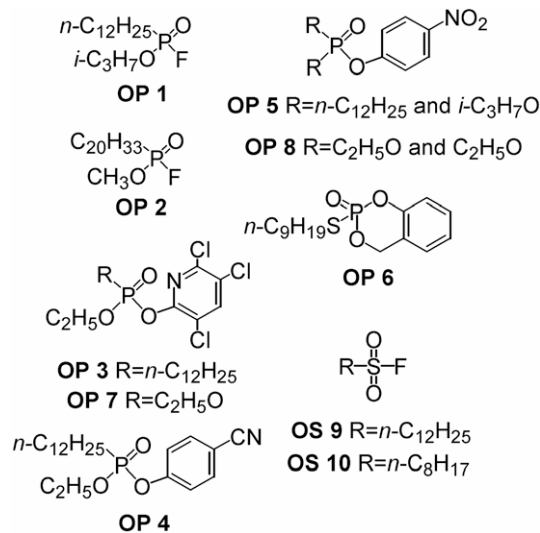
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The endocannabinoids (EC) 2-arachidonoylglycerol (2-AG) and anandamide (AEA) regulate a diverse array of neurological and metabolic functions and are altered by neuropathic pain, anxiety, neurodegeneration, obesity, and cardiovascular disorders.¹ 2-AG is a full agonist toward the cannabinoid receptor type 1 (CB1) and its signaling is terminated primarily by monoacylglycerol lipase (MAGL). AEA levels are regulated by fatty acid amide hydrolase (FAAH).^{2–4} Augmentation of EC signaling by blockade of 2-AG or AEA degradation (Scheme 1) is proposed as a therapeutic strategy. However, characterization of MAGL or 2-AG in brain and peripheral tissues is hindered by the paucity of systemic MAGL inhibitors and lack of a MAGL knockout mouse. Discovery of potent MAGL inhibitors is therefore essential in understanding the biochemical, physiological, and therapeutic roles of this enzyme.

Structural manipulation of organophosphorus (OP) and organosulfur (OS) compounds (Scheme 2) can potentially confer potency



Scheme 1. Endocannabinoids 2-AG and AEA are agonists toward CB1 and are metabolized by MAGL and FAAH, respectively, to arachidonic acid (AA).



Scheme 2. Organophosphorus (OP 1–8) and organosulfur (OS 9 and 10) compounds used in this study. In earlier literature OP 1, OP 2, OP 7, and OP 8 are referred to as IDFP, MAFP, chlorpyrifos oxon, and paraoxon, respectively.^{4–6}

and selectivity for MAGL and FAAH compared to other serine hydrolases. OP 1 and OP 2 are previously reported as highly potent MAGL and FAAH inhibitors.^{3,4} However, some OPs and OSs also displace CB1 agonist binding through an unknown mechanism.⁵

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This study reports structure–activity relationships of OPs and OSs with MAGL, FAAH, and CB1, and uses these tools to consider three interrelationships of the EC system components. The first is the *in vitro* potency for inhibiting MAGL, FAAH, and CB1 agonist binding as a predictor of *in vivo* behavioral effects and pharmacological profile. The second is the variation among tissues in their MAGL activity and differential regulation of 2-AG and AA levels. Finally, we consider the possibility that OP displacement of CB1 agonist binding is due to 2-AG in membranes which is metabolically stabilized by MAGL inhibition.

A library of 40 OPs and OSs, mostly prepared and optimized in the Berkeley laboratory,⁷ was tested for potency and selectivity as inhibitors of MAGL, FAAH, and CB1 agonist binding in mouse brain membranes.⁸ Five particularly potent OPs for all three targets were phosphonyl fluorides **1** and **2** and aryl phosphorus compounds **3–6**, all with long alkyl substituents [*n*-C₁₂H₂₅P, arachidonyl (C₂₀H₃₃P) or *n*-C₉H₁₉SP] (Table 1 and Supplementary data). One diethyl phosphate insecticide metabolite (OP **7**) was quite potent and another (OP **8**) was only moderately active. Two sulfonyl fluorides (**9** and **10**) with long alkyl chains were very potent on FAAH, moderately active on MAGL, and differed greatly in activity on CB1.

Eight potent *in vitro* inhibitors were administered intraperitoneally to mice at 10 mg/kg (OPs **1–6**) or 100 mg/kg (OS **9** and OS **10**) to determine if they were also effective *in vivo* in modulating behavior and brain 2-AG and arachidonic acid (AA) levels (Fig. 1). OP **1** and OP **4** were very effective *in vivo* in all respects, whereas OP **2** and OP **3** with similar *in vitro* potency to **1** and **4** were not effective *in vivo*. Thus, *in vitro* potency is not necessarily a predictor of *in vivo* activity with metabolic stability a likely contributor. OS **9** and OS **10** gave the same *in vivo* effects as OP **1** and OP **4** although at a 10-fold higher dose. Importantly, the OP- and OS-induced increase in brain 2-AG levels was always directly related to the lowering of brain free AA level.

2-AG and AA are important signaling molecules and intermediates not only in brain but also in other tissues.^{1,2,11} OP **1** at 10 mg/kg strongly inhibits brain MAGL activity, elevates 2-AG, and lowers AA,⁴ suggesting that it might also do so in other tissues (Fig. 2). 2-AG hydrolase activity was higher in brain than other tissues examined with 78–83% sensitive to OP **1** *in vivo* in brain, kidney, testes, pancreas, and liver and 92–99% OP **1**-sensitive *in vivo* in heart, spleen, and lung. The apparent coupling of 2-AG and AA levels was also examined. Among the tissues analyzed, brain, spinal cord, liver, spleen, and lung, but not kidney, testes, pancreas, or heart showed the possible co-dependence of 2-AG and AA pools (Fig. 2 and Supplementary data). Most tissues also had increased levels of other

Table 1
Inhibitory potencies of OPs and OSs for mouse brain MAGL and FAAH activities and CB1 agonist binding

Compound	IC ₅₀ ± SD (nM)		
	MAGL	FAAH	CB1
<i>Phosphonyl fluorides</i>			
OP 1	0.8 ± 0.2 ^a	3 ± 2 ^a	2 ± 1
OP 2	2.2 ± 0.3 ^a	0.10 ± 0.02 ^a	20 ^a
<i>Aryl phosphorus compounds</i>			
OP 3	0.14 ± 0.01	42 ± 12	14 ± 5
OP 4	0.07 ± 0.01	12 ± 3	4 ± 2
OP 5	0.28 ± 0.23	3 ± 1	12 ± 1
OP 6	0.31 ± 0.03 ^a	0.15 ± 0.01 ^a	
<i>Insecticide metabolites</i>			
OP 7	10 ± 4 ^a	40 ± 3 ^a	14 ± 3
OP 8	2300 ± 1100 ^a	540 ^a	1200 ^a
<i>Sulfonyl fluorides</i>			
OS 9	200 ± 75 ^a	2 ^a	7 ± 4
OS 10	140 ± 2 ^a	1.9 ± 0.2 ^a	1300 ± 300

^a Data derived from previous studies.^{3–5,9}

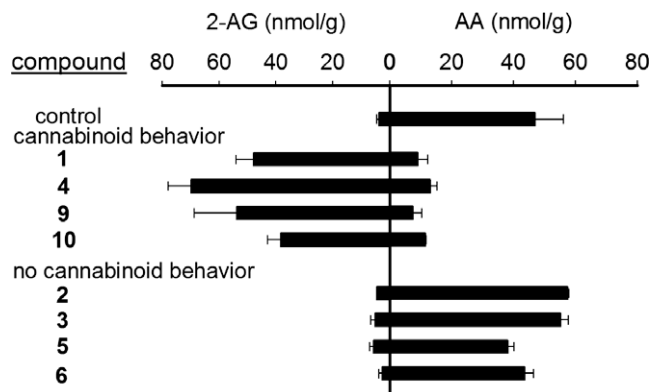


Figure 1. Modulatory action of OP MAGL inhibitors at 10 mg/kg and OS compounds **9** and **10** at 100 mg/kg on brain 2-AG and AA levels relative to cannabinoid behavior. Mice with cannabinoid behavior had >10 s latency in the bar test which assesses catalepsy.¹⁰ They also qualitatively had a flattened posture and remained motionless with their eyes open. Values are means ± SD, *n* = 3 mice/treatment group.

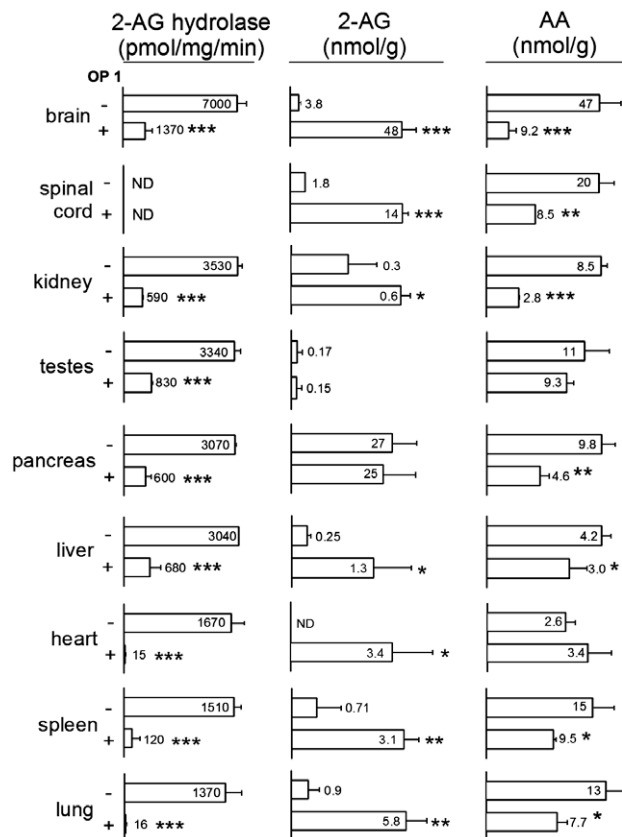


Figure 2. 2-AG hydrolase activities and 2-AG and AA levels in mice treated with OP **1** (10 mg/kg, ip 4 h).¹² Values are expressed as means ± SD, *n* = 3 mice/treatment group. ND, not determined. Significance expressed as **p* < 0.05, ***p* < 0.01, ****p* < 0.001 in unpaired Student's *t*-test.

monoacylglycerol species, that is, 1- and 2-palmitoylglycerol and 1- and 2-oleoylglycerol (Supplementary data). Beyond changes in glycerol esters and AA levels, OP **1** treatment also led to decreases in other unesterified fatty acid levels (palmitic, oleic, or stearic acid) in spinal cord, liver, and spleen, indicating off-target effects of OP **1** in these tissues. The heart interestingly showed increases in both oleic and stearic acids (Supplementary data).

Our results confirm the coordinate regulation of 2-AG and AA levels by MAGL in brain,⁴ and show that this regulation also exists in some peripheral tissues. These findings disfavor the current model in which AA in many tissues is released primarily through glycerophospholipid metabolism via multiple phospholipase A2 enzymes, notably cytosolic PLA2 (cPLA2), secretory PLA2 (sPLA2), and calcium-independent PLA2 (iPLA2). While there are multiple studies correlating increased PLA2 expression to pro-inflammatory outcomes, cPLA2^{-/-} mice (also deficient in sPLA2) have identical levels of plasma and brain nonesterified fatty acid levels and brain acyl-coenzyme A levels, albeit there were changes in esterified phospholipid levels.¹³ Although **OP 1** is not completely selective for MAGL, it does not inhibit iPLA2⁴ and the degree to which **OPs 1, 4, 9,** and **10** lower AA is equivalent to 2-AG elevation. MAGL inhibitors may help treat inflammatory diseases not only in brain but also in multiple peripheral tissues through the dual EC activation via 2-AG elevation and decreased eicosanoid signaling through AA reduction.

It was very surprising to find that many OPs are potent inhibitors of CB1 agonist binding in brain membranes. One possible mechanism is direct OP binding or phosphorylation of CB1 at the agonist or an allosteric site, and another is indirect by OP inhibition of MAGL or FAAH to elevate the levels of 2-AG or AEA, or both which then serve as the inhibitor. Three lines of evidence suggest that the OPs do not react directly with CB1. Agonist binding is OP-sensitive in brain membranes but not in recombinant expressed CB1 (eCB1)^{14,15} (Fig. 3a), indicating that some factor other than or in addition to CB1 is required. Covalently derivatized CB1 is not observed in brain membrane preparations labeled with a biotinylated fluorophosphonate probe under conditions in which phosphorylated MAGL and FAAH are readily evident.⁴ Inhibition by OP derivatization is expected to be essentially irreversible and noncompetitive with the agonist, whereas inhibition by **OP 7** gives an apparent competitive Scatchard plot (Fig. 3b and Supplementary data).¹⁶

An alternative hypothesis is that the OP inhibits MAGL and/or FAAH and elevates the 2-AG and/or AEA level which in turn blocks agonist binding (Schemes 1 and 3). CB1, assayed as agonist binding with [³H]CP55940, is highly sensitive to many OPs (Table 1, Fig. 3a and b, and Supplementary data) and **OP 1** potentiates the CB1 agonist action of 2-AG *in vitro* (measured by GTP γ S binding) (Fig. 3c).¹⁷ **OP 1** stimulates GTP γ S binding at much higher concentration (EC₅₀ 0.5 μ M) (similar to the 0.3 μ M EC₅₀ of 2-AG for CB1)¹⁷ than that required to displace agonist binding (IC₅₀ 2 nM) (Table 1) in similar preparations of brain membranes. The choice between MAGL/2-AG and FAAH/AEA as the target can be approached by OP sensitivity and specificity considerations, and by analysis for OP-induced elevations of EC levels. The OP sensitivity and specificity profiles correlate better for MAGL versus CB1 ($r^2 = 0.81$, $n = 27$) (Fig. 3d) than for FAAH versus CB1 ($r^2 = 0.68$, $n = 16$) (Supplementary data). Although AEA has higher CB1 affinity than 2-AG¹⁸, the ~1000-fold greater level of 2-AG may override the affinity difference. Importantly, there is sufficient accumulation of 2-AG on OP treatment to strongly inhibit the CB1 site (Fig. 3e).¹⁹ These results support lipid rafts²⁰ as an important compartment for 2-AG in its interactions with CB1 and MAGL. The weight of evidence favors OP action on CB1 initiated by MAGL inhibition rather than FAAH inhibition or direct on the receptor.

In conclusion, we report the discovery of several OP MAGL inhibitors with unprecedented *in vitro* potency (IC₅₀ < 1 nM), a subset of which is effective *in vivo* in dramatically raising brain 2-AG levels, leading to cannabinoid behavior. These inhibitors are attractive probes to uncover specific functions of MAGL and 2-AG in EC signaling *in vivo* both centrally and peripherally, and to investigate MAGL as a therapeutic target. The findings establish that MAGL and 2-AG, and not phospholipases and phospholipids,

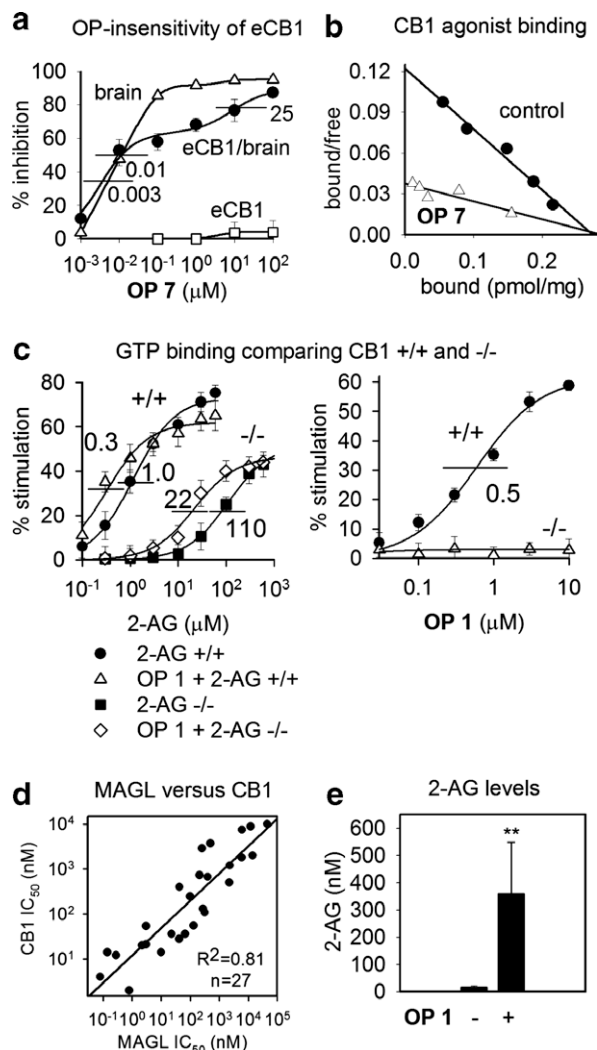
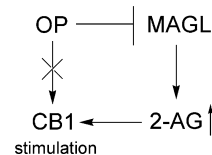


Figure 3. Mechanisms of OP action on brain CB1. (a) **OP 7** displaces [³H]CP55940 agonist binding in mouse brain membranes, but not in CB1 overexpressed in HEK293 cells (eCB1). The eCB1/brain curve used a mixture of 100 μ g eCB1 and 100 μ g brain membranes. IC₅₀ values (μ M) refer to brain (0.01) or components of eCB1/brain (0.003 and 25). (b) Scatchard plot for apparent competitive **OP 7** (100 nM) displacement of [³H]CP55940 agonist binding. (c) Stimulation of GTP binding by 2-AG, 2-AG plus **OP 1** (150 nM), or **OP 1** alone comparing CB1^{+/+} and CB1^{-/-} mouse brain membranes. (d) Similar OP sensitivity and specificity profiles for MAGL and CB1. (e) **OP 1** (10 mg/kg ip, 4h) significantly elevated brain membrane 2-AG levels. Values are means \pm SD, $n = 3-6$. Significance expressed as ** $p = 0.01$.



Scheme 3. Several lines of evidence are presented for indirect OP inhibition of CB1 agonist binding in brain membranes by inhibiting MAGL to elevate 2-AG that binds to CB1 rather than direct binding or phosphorylation of CB1.

regulate brain levels of free AA in multiple tissues. Finally, we propose a mechanism for OPs and other MAGL inhibitors to indirectly displace exogenous CB1 agonist binding in which elevated 2-AG levels, metabolically stabilized in brain membranes by MAGL inhibition, serve as the actual inhibitor.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.bmcl.2008.08.007](https://doi.org/10.1016/j.bmcl.2008.08.007).

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- MAGL activity was determined with either unlabeled 2-AG or [¹⁴C] 1-oleoylglycerol, FAAH activity with [³H]anandamide and CB1 agonist binding with [³H]CP55940 as described previously.^{3,4} The same IC₅₀ value (0.07 μ M) was found for **OP 4** in assays with 2-AG and [¹⁴C]1-oleoylglycerol (Supplementary data).
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- eCB1 overexpressed in HEK293 cells is not sensitive to **OP 1** or **OP 7** (up to 100,000 nM) although it displays appropriate [³H]CP55940 binding and sensitivity to WIN55212-2. Upon addition of brain membranes to eCB1, **OP 7**-sensitivity was partially restored.
- Kinetic experiments were performed as binding isotherms for **OP 7** displacement of [³H]CP55940 agonist binding (see Supplementary data). *K_d* (nM) 0.86 for control and 2.0 for **OP 7**. *B_{max}* (pmol/mg) 0.27 for control and 0.26 for **OP 7**.
- Guanosine-5-*O*-(γ -thio)-triphosphate (GTP γ S) binding was determined as previously described.⁴ Stimulation of GTP γ S binding by 2-AG is potentiated by preincubation with **OP 1** (150 nM) shifting the EC₅₀ of 2-AG from 1.0 to 0.3 μ M. Interestingly, there is significant 2-AG-mediated stimulation of GTP binding in CB1^{-/-} mouse brain, also potentiated by **OP 1**, indicating the possible existence of another cannabinoid receptor. **OP 1** alone at higher concentrations stimulates GTP γ S binding in CB1^{+/+} membranes (EC₅₀ 0.5 μ M), but not in CB1^{-/-} membranes, suggesting a possible direct stimulatory action of **OP 1** on CB1, but not on the other 2-AG-responsive receptor. This concentration is 600-fold higher than the IC₅₀ of MAGL and ~250-fold higher than the IC₅₀ of CB1 agonist binding.
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